NOVEL FULL-LENGTH GENOMIC RNA OF JAPANESE ENCEPHALITIS VIRUS, INFECTIOUS JEV cDNA THEREFROM, AND USE THEREOF

FIELD OF THE INVENTION

The present invention relates to the determination of an authentic Japanese encephalitis virus (JEV) genome RNA sequences, to construction of infectious JEV cDNA clones, and to utility of the clones or their derivatives for the purpose of therapeutic, vaccine, and diagnostic applications. In addition, the invention is also related to JEV vectors, e.g., for heterologous gene expression systems, genetic immunization, and transient gene therapy.

BACKGROUND

JEV is a member of the *Flaviviridae* family and is transmitted by mosquitoes. It is an important human pathogen that causes permanent neuropsychiatric sequelae and even fatal disease, especially in children (Tsai, *Vaccine*, 2000, 18(Suppl 2), 1-25; Solomon, *Neurological Infections and Epidemiology*, 1997, 2, 191-199; Umenai et al., *Bull. W.H.O.*, 1985, 63, 625-631). Up to 50,000 cases with a mortality rate of about 25%

are reported annually, and about half of the survivors exhibit permanent neuropsychiatric sequelae (Vaughn and Hoke, *Epidemiol. Rev.*, 1992, 14, 197-221; Burke and Leake, *Japanese encephalitis*, 1988, 63-92, CRC Press Publisher). JEV is distributed mostly in Asia from the former Soviet Union to India. In recent years, however, transmission of the virus has recently been observed in the southern hemisphere, indicating that this virus could become a worldwide public health threat (Hanna, et al., *Med. J. Aust.*, 1999, 170, 533-536; Hanna, et al., *Med. J. Aust.*, 1996, 165, 256-260; Mackenzie et al., *Arch. Virol.*, 1994, 136, 447-467).

JEV is a small-enveloped virus with a single-stranded, positive-sense RNA genome approximately 11 kb in length. The genome contains a single long open reading frame (ORF) flanked by 5' and 3' nontranslated regions (NTRs) that are important *cis*-acting elements for viral replication. The RNA genome of JEV has a type I cap structure at its 5'-terminus but lacks a poly(A) tail at its 3' terminus. The ORF is translated into a large polyprotein that is co- or posttranslationally processed into three structural and seven nonstructural proteins whose genes are arranged in the genome as follows: C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5 (Lindenbach and Rice, *Flaviviridae: The*

viruses and their replication, 2001, 991-1041, Lippincott Williams&Wilkins Publishers; Venugopal and Gould, *Vaccine*, 1994, 12, 966-975; Chamber et al., *Ann. Rev. Microbiol.*, 1990, 44, 649-688). Further information, for example, on the function of the majority of the JEV gene products and the molecular mechanisms involved in JEV replication, neurovirulence, and pathogenesis, is limited largely because of the lack of a reliable reverse genetics system.

Research investigating positive-sense RNA viruses has been considerably advanced by the development of the reverse genetics system. Here, infectious cDNA clones of the viral genome in question are constructed and become the templates for infectious RNA synthesis that generates synthetic viruses. There are two approaches, RNA-launched approach and DNA-launched approach, for the reverse genetics system. In the classical "RNA-launched" approach, cells are transfected with RNA transcripts made from the infectious cDNA clones, and the synthetic viruses are then recovered from these cells (Satyanarayana et al., *Proc. Natl. Acad. Sci. USA*, 1999, 96, 7433-7438; van Dinten et al., *Proc. Natl. Acad. Sci. USA*, 1997, 94, 991-996; Liljestrom and Garoff, *Biotechnology*, 1991, 9,

1356-1361; Rice et al., *New Biol.*, 1989, 1, 285-296, Rice et al., *J. Virol.*, 1987, 61, 3809-3819). In an alternative "DNA-launched" approach, synthetic viruses are generated by directly transfecting infectious cDNA clones into susceptible cells. This approach was first reported for poliovirus (Racaniello and Baltimore, *Science*, 1981, 214, 916-919), and has been adapted for alphaviruses (Schlesinger and Dubensky, *Curr. Opin. Biotechnol.*, 1999, 10, 434-439).

Both of these approaches have been used to construct infectious cDNA clones for many positive-sense RNA virus families, including coronaviruses, which have the largest RNA genomes (Almazan et al., *Proc. Natl. Acad. Sci. USA*, 2000, 97, 5516-5521). These clones have been invaluable in addressing many questions regarding the positive-sense RNA viruses. However, the construction of a full-length infectious cDNA clone for JEV has been hampered, largely because of the genetic instability of the cloned cDNA. Despite extensive efforts, a genetically stable full-length infectious cDNA molecular clone for JEV does not exist (Mishin et al., *Virus Res.*, 2001, 81, 113-123; Zhang et al., *J. Virol. Methods*, 2001, 96, 171-182; Sumiyoshi et al., *J. Infect. Dis.*, 1995, 171, 1144-1151; Sumiyoshi et al., *J. Virol.*, 1992, 66, 5425-5431).

Thus, the present inventors have disclosed the complete full-length nucleotide sequence of the JEV strain CNU/LP2, isolated from a pool of circulating mosquitoes in Korea. Based on this sequence, the present inventors also have developed a convenient and reliable reverse genetics system for JEV by synthesizing full-length infectious JEV cDNA molecular clones. The reverse genetics system based on the novel infectious JEV cDNA of the present invention can be effectively used for investigating the functions of JEV gene products and other molecular biological mechanisms related to replication, neurovirulence, and pathogenesis of JEV. Further, the present inventors have completed the present invention by confirming that the infectious JEV cDNA can be effectively used as a vector for the heterologous gene expression in a variety of ways.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

It is an object of the present invention to provide an authentic JEV genome RNA sequences, infectious JEV cDNA clones therefrom, and utility of the clones or their derivatives for novel gene

expression vectors.

To accomplish the above object,

1) The present invention provides an authentic JEV genome RNA sequences.

2) The present invention provides infectious JEV cDNA clones that are able to produce self-replicable JEV RNA transcripts.

3) The present invention provides a JEV-based vector.

4) The present invention provides a self-replicable RNA transcript synthesized from the above JEV-based vector.

5) The present invention provides a recombinant JEV virus obtained from cells transfected with a synthetic RNA transcript synthesized from the JEV-based vector.

6) The present invention provides a JEV-based expression vector.

7) The present invention provides a variety of strategies for expressing heterologous genes using the JEV-based expression vector.

Further features of the present invention will appear hereinafter.

I . The present invention provides an authentic JEV genome RNA sequences.

Korean isolate JEV genomic RNA of the present invention is composed of a 5'nontranslated region (NTR), a polypeptide coding region and a 3'NTR. Particularly, the full-length RNA genome is 10,968 bp in length and consists of a 95 bp 5'NTR followed by a 10,299 bp single open reading frame and terminated by a 574 bp 3'NTR.

According to the preferred embodiment of the present invention, the novel genomic RNA of JEV has a sequence represented by SEQ. ID. No 15. And the novel genomic RNA of the present invention also includes any sequence having 98% homology with JEV genomic RNA represented by SEQ. ID. No 15.

Korean isolate JEV of the present invention was isolated and purified from Korean JEV strain K87P39 by taking advantage of plaque-purification technique, and was named "JEV CNU/LP2" (see FIG. 1).

In order to determine the complete nucleotide sequence of CNU/LP2, a Korean isolate JEV, the present inventors amplified the entire viral RNA genome apart from the 5' and 3' termini using long reverse transcription-polymerase chain reaction (RT-PCR) and

yielded three overlapping cDNA products denoted JVF (nucleotide (nt) 1-3865), JVM (nt 3266-8170), and JVR (nt 7565-10893) (about 3.9, 4.9, and 3.3 kb in length, respectively) (see FIG. 2A).

The 3'-terminal sequence of CNU/LP2 viral RNA was analyzed after synthetic oligonucleotide T was ligated to it. Oligonucleotide T serves as a specific priming site for cDNA synthesis and PCR amplification (see FIG. 2B). Agarose gel electrophoresis revealed that the amplified products migrated as two bands, a larger band of approximately 700 bp and a smaller band of about 450 bp (see FIG. 2C). Both bands were purified and cloned, and 20 and 10 randomly picked clones containing the larger and the smaller bands, respectively, were sequenced. As has been documented for most of the fully sequenced JEV isolates, the present inventors found that all the clones with the larger insert (about 700 bp) terminated the viral genome with $-GATCT^{10968}$. In contrast, all the clones with the smaller insert (about 450 bp) showed the viral genome truncated at nt 10,684, resulting in a band 284 bp shorter. During assembly of the full-length JEV cDNA, the present inventors used the nucleotide sequences of the larger insert because the smaller insert did not contain 284 nucleotides at

the 3′ end of the viral genome.

The 5′-terminal sequence of CNU/LP2 viral RNA was examined after the cap structure at its 5′ end had been removed by incubation with tobacco acid pyrophosphatase. The resulting viral RNA was then self-ligated, and the 3′-5′ junction was subjected to cDNA synthesis and PCR amplification with a positive-sense primer for RT-PCR complementary to a sequence near the viral 3′ end (nt 10259-nt 10276) and a negative-sense primer corresponding to a sequence near the viral 5′ end (nt 164-nt 181) (see FIG. 2D). Agarose gel electrophoresis revealed the amplified products as a single band of about 850 bp (see FIG. 2E). The amplicons were cloned, and 12 randomly picked clones were sequenced. In all 12 clones, the -GATCT^{10968} of the viral 3′-terminal sequence was followed by the 5′-terminal sequence ^{1}AGAAGT- (see FIG. 2B and 2C).

Thus, the present inventors have determined the complete nucleotide sequence of the JEV CNU/LP2 isolate represented by SEQ. ID. No 15. The full-length RNA genome of JEV CNU/LP2 is 10,968 bp in length and consists of a 95 bp 5′NTR followed by a 10,299 bp single open reading frame and terminated by a 574 bp

3'NTR. The present inventors compared the complete nucleotide sequence of the CNU/LP2 isolate with sequences of all 26 JEV strains (Ishikawa, K94P05, FU, CH2195LA, CH2195SA, RP-2ms, RP-9, CH1392, T1P1, YL, JaGAr01, HVI, TC, TL, Beijing-1, Ling, Vellore P20778, p3, SA14-14-2, SA(A), SA14-12-1-7, SA14-2-8, SA14, SA(V), GP78, and JaOArS982) available in GenBank database. Such informations concerning viral strains used for the comparison as isolation regions, isolation years, sources and GenBank accession numbers are briefly stated hereinafter (see Table 1).

<Table 1>

Geographic location	Year	Strain	Source	GenBank accession number
Australia	1995	FU	Human serum	AF217620
China	1954	SA14	Mosquito	U14163
		SA14-14-2	SA14 derivative	AF315119
		SA14-12-1-7	SA14 derivative	AF416457
		SA14-2-8	SA14 derivative	U15763
		SA(V)	SA14 derivative	D90194
		SA(A)	SA14-14-2 derivative	D90195
	1949	Beijing-1	Human brain	L48961
	1949	p3	Mosquito	U47032
India	1978	GP78	Human brain	AF075723

	1958	Vellore P20778	Human brain	AF080251
Japan	1982	JaOArS982	Mosquito	M18370
	IU	Ishikawa	IU	AB051292
	1959	JaGAr01	Mosquito	AF069076
Korea	1994	K94P05	Mosquito	AF045551
	1987	CNU/LP2	Mosquito	This invention
Taiwan	1997	T1P1	Mosquito	AF254453
	1994	CH2195LA	CH2195 derivative	AF221499
	1994	CH2195SA	CH2195 derivative	AF221500
	1990	CH1392	Mosquito	AF254452
	1985	RP-2ms	Mosquito	AF014160
	1985	RP-9	Mosquito	AF014161
	1965	Ling	Human brain	L78128
	IU	YL	IU	AF486638
	IU	TC	Mosquito	AF098736
	IU	TL	Mosquito	AF098737
	IU	HVI	Mosquito	AF098735

IU : Information unavailable

From the comparison of the nucleotide sequence of the CNU/LP2 isolate with nucleotide sequences of other JEV strains, it was shown that the JEV isolate CNU/LP2 genome shared various degrees of sequence similarity with these other genomes [89.0% (Ishikawa), 89.1% (K94P05), 89.3% (FU), 95.8% (CH2195LA), 95.9% (CH2195SA), 97.1% (RP-2ms), 97.2% (RP-9), 97.3%

(CH1392), 97.3% (T1P1), 97.0% (YL), 97.4% (JaGAr01), 97.1% (HVI), 96.9% (TC), 96.7% (TL), 96.4% (Beijing-1), 96.3% (Ling), 96.0% (Vellore P20778), 97.1% (p3), 97.4% (SA14-14-2), 97.5% (SA(A)), 97.5% (SA14-12-1-7), 97.7% (SA14-2-8), 97.9% (SA14), 97.9% (SA(V)), 96.3% (GP78), and 97.1% (JaOArS982)] (see Table 2). Therefore, the nucleotide sequences of JEV viral genomic RNA having over 98% sequence similarity with the nucleotide sequence of the present invention represented by SEQ. ID. NO 15 can be included in the category of the claim of the present invention.

<Table 2>

Isolate	% sequence identity[a]																												
	Ishikawa	K94P05	FU	CH2195LA	CH2195SA	RP-2ms	RP-9	CH1392	T1P1	YL	JaGAr01	HVI	TC	TL	Beijing-1	Ling	Vellore P20778	p3	SA14-14-2	SA(A)	SA14-12-1-7	SA14-2-8	SA14	SA(V)	CNIU/LP2	GP78	JaOArS982	WNV
Ishikawa		97.0	90.1	88.3	88.3	88.9	89.0	89.1	89.1	88.9	89.2	89.0	88.9	88.8	88.9	88.8	88.8	89.1	88.8	88.9	88.9	89.1	89.2	89.2	88.0	88.8	88.9	69.0
K94P05	97.7		89.8	88.6	88.6	89.2	89.3	89.4	89.4	89.1	89.5	89.2	89.1	89.0	89.0	89.0	88.9	89.4	89.1	89.2	89.2	89.3	89.5	89.5	89.1	89.0	89.5	68.7
FU	97.7	97.0		88.9	88.9	89.3	89.4	89.4	89.4	89.2	89.6	89.4	89.2	89.1	89.3	89.0	89.1	89.4	89.1	89.2	89.2	89.3	89.6	89.8	89.3	88.7	89.4	69.5
CH2195LA	97.7	97.0	89.9		99.9	96.2	96.3	96.3	96.3	96.1	96.5	96.2	95.9	95.8	95.6	95.7	95.1	96.3	96.6	96.7	96.7	96.9	97.1	97.1	95.8	95.7	97.3	69.4
CH2195SA	97.1	96.5	99.0	99.0		96.3	96.3	96.3	96.3	96.1	96.5	96.2	95.9	95.8	95.6	95.7	95.1	96.3	96.6	96.7	96.7	96.9	97.1	97.1	95.9	95.7	97.3	69.5
RP-2ms	97.5	96.9	99.4	99.4	99.6		99.9	99.5	99.5	99.3	99.4	99.8	99.2	99.1	97.2	97.1	96.6	97.9	98.0	98.1	98.1	98.3	98.5	98.5	97.1	96.9	97.7	69.4
RP-9	97.6	96.9	99.5	99.5	99.9	99.7		99.6	99.5	99.3	99.5	99.8	99.2	99.2	97.2	97.2	96.7	98.0	98.1	98.1	98.1	98.3	98.5	98.5	97.2	96.9	97.6	69.4
CH1392	97.8	97.2	99.7	99.7	99.1	99.8	99.7		99.9	99.5	99.7	99.0	99.3	99.3	97.3	97.3	96.8	98.2	98.2	98.3	98.3	98.4	98.6	98.7	97.3	97.0	97.9	69.4
T1P1	97.5	96.8	99.3	99.3	99.7	99.1	99.2	99.4		99.5	99.7	99.0	99.3	99.3	97.3	97.3	96.8	98.1	98.2	98.2	98.3	98.4	98.6	98.6	97.3	97.0	97.8	69.4
YL	97.4	96.7	99.2	99.2	99.7	99.1	99.2	99.4	99.0		99.4	99.8	99.1	99.0	97.1	97.1	96.5	97.9	98.0	98.0	98.0	98.2	98.4	98.4	97.0	96.8	97.6	69.2
JaGAr01	97.1	96.4	96.9	96.9	96.2	96.7	96.8	99.1	96.6	96.7		99.1	99.4	99.4	97.4	97.4	96.9	98.3	96.3	98.4	98.4	98.5	98.6	98.8	97.4	97.1	98.0	69.5
HVI	97.2	96.5	98.9	98.9	99.3	99.8	98.9	99.1	99.7	99.8	99.7		99.6	99.4	97.2	97.2	96.7	98.1	99.1	98.1	98.1	98.3	98.5	98.5	97.1	96.9	97.7	69.4
TC	97.0	96.4	98.8	98.8	99.2	98.7	98.8	99.0	98.8	98.5	98.2	98.4		99.0	97.2	97.1	96.4	97.7	97.8	97.9	97.8	98.1	98.3	98.3	96.9	96.7	97.4	69.5
TL	97.2	96.6	99.0	99.0	99.4	96.8	96.9	99.2	99.8	99.7	99.4	99.5	99.7		97.0	96.8	96.1	97.5	97.8	97.7	97.7	97.9	98.1	98.1	96.7	96.5	97.2	69.3
Beijing-1	97.3	96.6	99.0	99.0	99.5	98.9	99.0	99.2	99.8	99.7	99.4	99.6	99.2	99.3		99.1	96.7	97.4	97.2	97.2	97.3	97.5	97.6	97.6	96.4	96.1	97.0	69.5
Ling	97.4	96.7	99.1	99.1	99.5	99.0	99.1	99.3	99.9	99.9	99.6	99.7	99.0	99.2	99.2		96.7	97.3	97.1	97.2	97.2	97.4	97.6	97.6	96.3	96.2	97.2	69.5
Vellore P20778	97.7	97.1	99.5	99.5	99.9	99.4	99.5	99.7	99.9	99.2	98.9	99.1	99.1	99.2	99.3	99.4		96.8	96.8	96.7	96.7	96.9	97.1	97.1	96.0	95.8	96.4	69.5
p3	97.8	97.1	99.5	99.5	98.9	99.4	99.5	99.7	99.4	99.3	99.0	99.2	99.0	99.2	99.2	99.5	99.8		98.2	98.3	98.3	98.5	98.7	98.7	97.1	97.1	97.8	69.5
SA14-14-2	97.9	97.1	99.5	99.5	98.9	99.3	99.4	99.7	99.2	99.2	98.9	99.0	98.8	99.0	99.1	99.2	99.6	99.8		99.8	99.6	99.4	99.4	99.4	97.4	97.3	98.1	69.4
SA(A)	97.1	96.8	98.8	98.8	98.2	98.7	98.8	99.0	98.8	98.5	98.2	98.3	98.1	98.3	98.4	98.5	98.8	98.9	98.9		99.8	99.4	99.4	99.4	97.5	97.4	98.2	69.4
SA14-12-1-7	97.2	96.8	98.9	98.8	98.3	98.7	98.9	99.1	98.7	98.6	98.3	98.4	98.2	98.4	98.5	98.5	99.0	99.0	98.9	99.7		99.4	99.5	99.5	97.5	97.4	98.2	69.4
SA14-2-8	97.7	97.3	99.4	99.4	98.8	99.3	99.4	99.6	99.2	99.2	98.8	98.9	98.8	98.9	99.0	99.2	99.5	99.8	99.5	99.1	99.2		99.6	99.6	97.7	97.6	98.3	69.5
SA14	97.5	96.7	99.0	99.0	98.4	98.8	99.0	99.2	98.9	98.8	98.6	98.7	98.4	98.5	98.6	98.7	99.1	99.2	99.2	98.5	98.5	99.2		99.9	97.9	97.8	98.6	69.6
SA(V)	97.3	96.6	98.9	98.9	98.3	98.7	98.8	99.1	98.7	98.8	98.4	98.5	98.2	98.4	98.5	98.6	98.9	99.0	99.0	98.3	98.4	99.0	99.5		97.9	97.8	98.6	69.6
CNIU/LP2	97.4	96.7	98.9	98.9	98.2	98.7	98.8	99.1	98.6	98.6	98.2	98.4	98.2	98.4	98.5	98.6	98.9	99.0	98.4	98.4	99.0	98.6	98.5		98.3	97.1	98.5	
GP78	97.0	96.4	98.6	98.6	98.0	98.5	98.6	98.8	98.5	98.4	98.0	98.1	98.0	98.2	98.3	98.3	98.7	98.7	98.7	98.1	98.2	98.7	98.2	98.2	98.2		97.2	69.6
JaOArS982	97.5	96.8	97.8	97.8	97.2	97.7	97.8	98.0	97.8	97.6	97.2	97.3	97.1	97.3	97.4	97.5	97.9	97.9	98.0	97.3	97.4	97.9	97.6	97.4	97.5	97.1		69.8
WNV	76.2	75.8	76.6	76.8	76.1	76.4	76.5	76.7	76.5	76.5	76.3	76.4	76.1	76.2	76.3	76.4	76.6	76.6	76.8	76.2	76.3	76.7	76.6	76.5	76.4	76.5	76.7	

[a]The percent nucleotide sequence identities of the complete genomes are presented at the upper right. The percent amino acid sequence identities of the complete genomes are shown in the lower left. The percentages of CNU/LP2 sequence identities are indicated in boldface type.

In addition to determine the nucleotide sequence of polypeptide coding region of JEV, the nucleotide sequences of 5′ and 3′NTRs including *cis*-acting elements involved in the regulation of viral replication, transcription, and translation of the virus were also determined by taking advantage of molecular biological approaches. The importance of both regions have been supported by some of earlier studies reporting that both the 5′- and 3′-terminal regions are required for the initiation of flavivirus RNA replication *in vitro* (You and Padmanabhan, *J. Biol. Chem.*, 1999, 274, 33714-33722) and *in vivo* (Khromykh et al., *J. Virol.*, 2001, 75, 6719-6728). Especially, ^{1}AGAAGT- and -GATCT^{10968}, which were proved to be the nucleotide sequence of 5′- and 3′-terminal regions of JEV CNU/LP2 in the present invention, are highly expected to play an important role in self-replication of the virus.

The present inventors proved through the experiments illustrated hereinafter that infectious synthetic JEV could be produced when cells were transfected with a synthetic RNA transcript having a full-length nucleotide sequence of JEV, and further, the inventors are the first to prove the function of the complete full-length nucleotide sequence which is necessary for JEV self-replication.

II . The present invention provides infectious JEV cDNA clones that are able to produce a self-replicable JEV RNA transcripts.

The infectious JEV cDNA clones of the present invention was synthesized with a nucleotide sequence represented by SEQ. ID. No 15 or nucleotide sequences of full-length JEV genomic RNA having over 98% sequence similarity therewith, and was used as a template for the synthesis of self-replicable JEV RNA transcript through *in vitro* transcription. In order to construct the full-length JEV cDNA clones, a viral genomic RNA including 5′- and 3′-terminal regions should be amplified by RT-PCR and then the obtained overlapping cDNAs were sequentially assembled.

In order to produce a full-length synthetic JEV RNA transcript through *in vitro* runoff transcription reaction, SP6 or T7 promoter transcription start site was located at the front of 5′-end of JEV genomic RNA and a unique restriction endonuclease recognition site was located at the end of the viral genome (see FIG. 3A). In the preferred embodiment of the present invention, three SP6-driven full-length JEV cDNAs and three T7-driven full-length JEV cDNAs were constructed by using three overlapping JEV cDNAs (JVF, JVM and JVR) and two additional cDNAs; one is corresponding to 5′-terminal region including SP6 or T7 promoter sequence and the other is corresponding to 3′-terminal region including *Xho* I and *Xba* I recognition sequence as a runoff site (see FIG. 3B and 3C). However, it is a common knowledge for the people in this field that other promoters but the above two promoters can be used as well. The full-length JEV cDNA developed in the present invention uses *Xho* I and *Xba* I as a runoff site but other restriction enzymes can be used as commonly known.

The JEV cDNA clones of the present invention are constructed by producing subclones containing many overlapping cDNAs using the bacterial artificial

chromosome (BAC) plasmid pBeloBAC11 as a vector and sequentially linking those subclones into the full-length JEV cDNAs.

In the preferred embodiment of the present invention, the present inventors provide one set of three JEV cDNA clones having SP6 promoter and represented by SEQ. ID. No 43, No 44, and No 45, respectively. In addition, the present inventors also provide the other set of three JEV cDNA clones having T7 promoter and represented by SEQ. ID. No 46, No 47, and No 48, respectively (see FIG. 3B and 3C). To ensure that the 3' end of the viral genome after runoff transcription would be close to authentic, in all cases, the present inventors placed a unique restriction endonuclease recognition site, either *Xho* I or *Xba* I, at the end of the viral genome (see FIG. 3B and 3C).

III. The present invention provides a JEV-based vector.

The vector of the present invention is characterized by including a full-length infectious JEV cDNA. In the preferred embodiment of the present invention, the inventors provide vectors 'pBAC^{sp6}/JVFL/*Xho*I', 'pBAC^{sp6}/JVFLx/*Xho*I', and 'pBAC^{sp6}/JVFLx/*Xba*I' which all have SP6 promoter and

each is represented by SEQ. ID. No 43, No 44, and No 45, and also vectors 'pBAC^{T7}/JVFL/*Xho*I', 'pBAC^{T7}/JVFLx/*Xho*I', and 'pBAC^{T7}/JVFLx/*Xba*I' which all have T7 promoter and each is represented by SEQ. ID. No 46, No 47, and No 48. The present inventors deposited two most efficient vectors of the above, pBAC^{T7}/JVFLx/*Xba*I and pBAC^{SP6}/JVFLx/*Xba*I, at Gene Bank of Korea Research Institute of Bioscience and Biotechnology (KRIBB) on October 2, 2002 (Accession No: KCTC 10346BP, KCTC 10347BP).

IV. The present invention provides a self-replicable RNA transcript synthesized from the above JEV-based vector.

For *in vitro* runoff transcription, JEV cDNA templates were linearized by digestion with *Xho* I or *Xba* I which is engineered for run-off site right behind 3'-terminal region of the viral genome (see FIG. 3). SP6 polymerase runoff transcription of the two *Xho* I-linearized plasmids (pBAC^{SP6}/JVFL/*Xho*I and pBAC^{SP6}/JVFLx/*Xho*I) in the presence of the $m^7G(5')ppp(5')A$ cap structure analog yielded capped synthetic RNAs containing three nucleotides (CGA) of virus-unrelated sequence at their 3' ends (see FIG. 3B). This is the result of copying the 5' overhang left by

the *Xho* I digestion. Similarly, SP6 polymerase runoff transcription of the *Xba* I-linearized pBAC^{SP6}/JVFLx/*Xba*I plasmid in the presence of the $m^7G(5')ppp(5')A$ cap structure analog produced capped synthetic RNAs with four nucleotides (CTAG) of virus-unrelated sequence at their 3′ ends (see FIG. 3B).

The present inventors have performed infectious center assay to measure the specific infectivity of the synthetic JEV RNA transcripts. As a result, when susceptible BHK-21 cells were transfected with the synthetic RNA transcripts, all were highly infectious ($3.4\text{-}4.3 \times 10^5$ PFU/μg) (see Table 3). Similar results ($2.9\text{-}3.8 \times 10^5$ PFU/μg) were also obtained with synthetic RNAs transcribed from the T7-driven cDNA constructs by T7 polymerase runoff transcription (see Table 3).

It has been reported that for some flaviviruses, the presence of virus-unrelated sequences at the 3′ end of synthetic RNAs transcribed from infectious cDNA diminishes or abrogates their specific infectivity (Yamshchikov et al., *Virology*, 2001, 281, 294-304). Based on this report, the present inventors generated synthetic RNAs lacking virus-unrelated sequences at their 3′ends and compared their specific infectivities.

Particularly, the present inventors generated synthetic RNAs lacking the unrelated sequences by treating the *Xba* I-linearized pBAC^{sp6}/JVFLx/*Xba*I plasmid with mung bean nuclease (MBN) prior to the transcription reaction, which removed the four excess nucleotides of CTAG. To verify MBN activity, *Xba* I-linearized and MBN-treated pBAC^{SP6}/JVFLx/*Xba*I plasmid was self-ligated, and its viral 3′ end was sequenced, demonstrating removal of the four excess nucleotides of CTAG. RNA transcripts from *Xba* I-linearized and MBN-treated pBAC^{SP6}/JVFLx/*Xba*I and pBAC^{T7}/JVFLx/*Xba*I (pBAC^{SP6}/JVFLx/*Xba*I^{MBN}, see FIG. 3B and pBAC^{T7}/JVFLx/*Xba*I^{MBN}, see FIG. 3C) both had increased specific infectivities compared to the untreated transcripts. Precisely, the specific infectivity of RNAs transcribed from pBAC^{SP6}/JVFLx/*Xba*I^{MBN} was estimated to be 3.1 x 10^6 PFU/μg, approximately 10-fold higher than the specific infectivity (3.4 x 10^5 PFU/μg) of the unmodified template (see Table 3, infectivity). The RNAs derived from pBAC^{T7}/JVFLx/*Xba*I also had increased specific infectivity after MBN modification (2.7 x 10^6 PFU/μg) (see Table 3, infectivity). Therefore, the present inventors confirmed that the authentic 3′ end of the JEV genome should be present to ensure highly infectious synthetic JEV RNA transcripts are generated.

Thus, the infectious JEV cDNA clones of the present invention could be used as templates for runoff transcription that generated highly infectious synthetic RNAs with a specific infectivity of 10^5 to 10^6 PFU/μg.

Previous attempts (Mishin et al., *Virus Res.*, 2001, 81, 113-123; Zhang et al., *J. Virol. Methods*, 2001, 96, 171-182; Sumiyoshi et al., *J. Infect. Dis.*, 1995, 171, 1144-1151; Sumiyoshi et al., *J. Virol.*, 1992, 66, 5425-5431) to assemble a full-length infectious JEV cDNA were all failed because of the genetic instability of cloned JEV cDNA. One study attempted to overcome this problem by designing a system in which the template would be generated by *in vitro* ligation of two overlapping JEV cDNAs (Sumiyoshi et al., *J. Virol.*, 1992, 66, 5425-5431). This template was then used to synthesize infectious RNA transcripts *in vitro*. However, the specific infectivity of these transcripts was about 100 PFU/μg, which was too low to make this system useful for molecular and genetic analyses of virus biology (Sumiyoshi et al., *J. Virol.*, 1992, 66, 5425-5431).

In the present invention, the present inventors were able to overcome the genetic instability of JEV

cDNA by cloning it into a BAC plasmid that is maintained at one or two copies in *E. coli*. The genetic structure and functional integrity of the infectious cDNA plasmid remained stable for at least 180 generations during its propagation in *E. coli* (see FIG. 7). So, the present inventors settled the problem of genetic instability of making full-length infectious JEV cDNA by introducing BAC, and further had skills to treat the synthetic infectious JEV cDNA stably.

It is important to produce full-length infectious JEV cDNA that, in *in vitro* transcription, would generate RNA transcripts with authentic 5′ and 3′ ends because several studies have shown that both the 5′- and 3′-terminal regions are needed for the initiation of flavivirus RNA replication *in vitro* (You and Padmanabhan, *J. Biol. Chem.*, 1999, 274, 33714-33722) and *in vivo* (Khromykh et al., *J. Virol.*, 2001, 75, 6719-6728). To achieve this objective, the present inventors adapted approaches used previously for other flaviviruses (van der Werf et al., *Proc. Natl. Acad. Sci. USA*, 1986, 83, 2330-2334; Rice et al., *New Biol.*, 1989, 1, 285-296). The cap structure in JEV genomic RNA is followed by the dinucleotide AG, an absolutely conserved feature of the flaviviruses (Rice,

Flaviviridae: The viruses and their replication, 1996, 931-960, Lippincott-Raven Publisher). The authenticity of the 5′ end was ensured by placing either the SP6 or the T7 promoter transcription start at the beginning of the viral genome. Incorporating the $m^7G(5')ppp(5')A$ cap structure analog in the SP6 or T7 polymerase-driven transcription reactions (Contreras et al., *Nucleic Acids Res.*, 1982, 10, 6353-6362), the present inventors synthesized capped RNA transcripts with authentic 5′ ends that were highly infectious upon transfection into susceptible cells. In addition, incorporating the $m^7G(5')ppp(5')G$ cap structure analog in the SP6 or T7 polymerase-driven transcription reactions (Contreras et al., *Nucleic Acids Res.*, 1982, 10, 6353-6362) places an unrelated extra G nucleotide upstream of the dinucleotide AG. As reported earlier (Rice et al., *New Biol.*, 1989, 1, 285-296), the present inventors did find that the extra nucleotide was lost from the genomic RNA of the recovered JEV progeny. Furthermore, the present inventors did not observe that the infectivity or the replication of synthetic RNAs transcribed from infectious cDNA templates was altered if the inventors added the extra nucleotide.

The dinucleotide CT located at the 3′ end of JEV RNA is absolutely conserved among the flaviviruses

(Rice, *Flaviviridae*: The viruses and their replication, 1996, 931-960, Lippincott-Raven Publisher). This suggests that these nucleotides are important in viral replication and that transcripts from infectious cDNAs must have authentic 3′ ends. Thus, the present inventors designed our reverse genetics system for JEV so that the synthetic RNA would be terminated with the authentic 3′ ends. Indeed, the present inventors showed that RNA transcripts with authentic 3′ ends were 10-fold more infectious than transcripts with three or four virus-unrelated nucleotides hanging on their 3′ ends.

V. The present invention provides a recombinant JEV virus obtained from cells transfected with a synthetic RNA transcript synthesized from the JEV-based vector.

In the present invention, synthetic JEV viruses produced from the cells transfected with JEV RNA transcripts synthesized from full-length infectious JEV cDNAs were produced. Transfected cells showed strong cytopathic effect induced by JEV virus infection and all the synthetic viruses were indistinguishable from the CNU/LP2 parental virus in terms of plaque morphology, cytopathogenicity, growth kinetics, protein

expression and RNA accumulation (see FIG. 5). Furthermore, recombinant JEV virus mutants could be produced by inducing site-directed mutation on a specific region of JEV cDNA, indicating that the infectious JEV cDNA can be manipulated in *E. coli*. Thus, the reverse genetics system using the infectious JEV cDNAs of the present invention can be effectively used for the genetic studies on the replication mechanism of JEV genome.

VI. The present invention provides a JEV-based expression vector.

The present invention provides the use of JEV cDNA as a novel expression vector in a variety of cell types. Alphaviruses, which are also RNA viruses, can replicate in a variety of commonly used animal cells and thus have been successfully exploited as eukaryotic expression vectors in cell culture and *in vivo* (Agapov et al., *Proc. Natl. Acad. Sci. USA*, 1998, 95, 12989-12944; Frolov et al., *Proc. Natl. Acad. Sci. USA*, 1996, 93, 11371-11377; Schlesinger, *Trends Biotechnol.*, 1993, 11, 18-22). It was reported that JEV, like the alphaviruses, is also able to replicate in a wide variety of primary and continuous cell cultures from humans, mice, monkeys, pigs, and hamsters (Burke and

Monath, *Flaviviruses*, 2001, 1043-1125, Lippincott Williams&Wilkins Publishers). This suggests that JEV could be useful as a vector for the expression of heterologous genes in a variety of different cells. When a full-length infectious JEV cDNA is used as an expression vector, in which heterologous genes are inserted, RNA transcripts having heterologous genes are produced by *in vitro* transcription reaction. Those transcripts can self-replicate as they are transfected into cells, so that lots of foreign proteins can be produced.

An expression cassette is preferably inserted at the beginning of JEV 3'NTR for the expression of a heterologous gene. A deletion of 9-25 bp exists at the beginning of the viral 3'NTR in CNP/LP2 and three other fully sequenced JEV strains (Williams et al., *J. Gen. Virol.*, 2000, 81, 2471-2480; Nam et al., *Am. J. Trop. Med. Hyg.*, 2001, 65, 388-392; Jan et al., *Am. J. Troop. Med. Hyg.*, 1996, 55, 603-609), suggesting that this may be a good site to insert the foreign genes. Thus, the infectious JEV cDNA developed by the present invention can act as a vector for rapid expression of heterologous genes in a variety of cells including mammalian cells.

VII. The present invention provides a variety of strategies for expressing heterologous genes using the JEV-based expression vector.

It is a function of the expression vector to deliver heterologous genes of interest into cells for the expression of those genes. In the present invention, the full-length infectious JEV cDNA has been demonstrated to act as a heterologous gene expression vector in a variety of cell types including mammalian cells.

Here, the present inventors also describe a heterologous gene expression system based on the full-length infectious JEV cDNA, which serves as a BAC (Yun et al., *J. Virol.*, 2003, 77, 6450-6465). As a transient expression system, JEV offers several advantages: (*i*) high titers of the virus are rapidly produced, (*ii*) the virus infects a wide range of host cells, including insect and mammalian cell types, (*iii*) the genetically stable infectious cDNA is available and readily manipulable, and (*iv*) the cytoplasmic replication of the RNA genome minimizes the possibility of its integration into the host's genome and the consequent undesirable mutagenic consequences.

The present inventors demonstrated here that the JEV-based system can be used to express foreign genes in three different ways. One involves infectious recombinant vector RNAs/viruses encoding the foreign gene, the second involves the production of a viral replication-competent but propagation-deficient JEV viral replicon vector RNA. The third involves the use of packaging systems for viral replicon particle (VRP) formation. Thus, the present inventors have shown here that the JEV system can be used to produce a JEV virus/infectious RNA/replicon RNA/VRP vector that will rapidly express foreign genes of interest in a wide variety of mammalian cell types.

The basic method for the expression of heterologous genes using the infectious or replicon JEV cDNA vectors of the present invention is composed of the following steps:

1) Preparing a recombinant JEV cDNA expression vector by inserting heterologous genes into the infectious or relicon JEV cDNA vector;

2) Producing a JEV RNA transcript from the above recombinant JEV cDNA expression vector;

3) Preparing a transformant by transfecting host cells with the above JEV RNA transcript; and

4) Expressing foreign proteins by culturing the above transformant.

The present inventors produced full-length infectious recombinant JEV cDNAs expressing green fluorescent protein (GFP), an enhanced version of GFP (EGFP), luciferase (LUC), and LacZ genes and the dominant selective marker puromycin *N*-acetyltransferase (PAC), which confers resistance to the drug puromycin, according to the method explained hereinbefore (see Fig. 8 and 9). BHK-21 cells were transfected with JEV RNA transcripts transcribed from the recombinant JEV cDNAs. GFP, EGFP, LUC, LacZ and PAC expression is shown in Fig. 8 and 10. In addition, recombinant infectious JEV viral particles containing those heterologous genes were prepared from culture supernatants. The expression of those heterologous genes was further investigated after infecting various animal cell lines (BHK-21, Vero, NIH/3T3, ST, HeLa, MDCK, CRFK, B103 and SHSY-5Y), which have been generally used in the field of biology and medicine, with the recombinant viruses. As a result, GFP or LUC gene inserted in virus genome was expressed in all cells tested (see Table 4). Thus, it was confirmed that recombinant JEV cDNAs, JEV RNA transcripts, and recombinant JEV viral paticles could

be effectively used as a vector for expression of foreign heterologous genes in a variety of cell types.

To independently express foreign genes using the JEV RNA replication machinery, the present inventors generated a panel of self-replicating self-limiting viral replicons by deleting one, two, or all of the viral structural genes, which meet stringent safety concerns (Fig. 11A). These viral replicons were capable of initiating replication and gene expression upon RNA transfection (see Fig. 11B and 11C).

The utility of the JEV replicon-based expression vectors was further elaborated by developing a panel of stable replicon packaging cell lines (PCLs) that would constitutively express all JEV viral structural proteins (C, prM, and E) *in trans* (see Fig. 12). These PCLs allowed the *trans*-complementation of the efficient packaging of JEV viral replicons. Thus, these PCLs were shown to be useful for efficiently producing high titer viral VRPs upon introducing JEV viral replicons (see Fig. 12).

The present inventors also showed that infectious JEV recombinant viral RNAs encoding heterologous genes

up to 3 kb can be packaged into the viral particles. By the choice of JEV viral replicon vectors such as JEV/Rep/ΔC+ΔprM+ΔE and JEV/Rep/NS1, it was estimated that a foreign gene of at least 5 kb could be packaged into the JEV VRPs. It will be of interest to examine the upper size limit of the foreign sequences that can be packaged in the JEV virion. This may be an important issue if one wishes to express lengthy genes such as cystic fibrosis transmembrane conductance regulator, whose coding sequence is approximately 4.5 kb (Flotte et al., *J. Biol. Chem.*, 1993, 268, 3781-3790). In addition, a large packaging capacity of JEV viral replicons would be useful if one wishes to add two or more expression units (Thiel et al., *J. Virol.*, 2003, 77, 9790-9798; Agapov et al., *Proc. Natl. Acad. Sci. USA*, 1998, 95, 12989-12994). In the case of the adeno-associated virus-based vector, its packaging capacity has been elegantly expanded to bypass its natural size limitation (Duan et al., *Nat. Med.*, 2000, 6, 595-598; Yan et al., *Proc. Natl. Acad. Sci. USA*, 2000, 97, 6716-6721), which shows that it may be possible to expand the packaging capabilities of JEV viral replicons in a similar manner.

As with other RNA virus-derived vectors (Agapov et

al., *Proc. Natl. Acad. Sci. USA*, 1998, 95, 12989-12994; Pushko et al., *Virology*, 1997, 239, 389-401; Berglund et al., *Nat. Biotechnol.*, 1998, 16, 562-565; Basak et al., *J. Interferon Cytokine Res.*, 1998, 18, 305-313; Barclay et al., *J. Gen. Virol.*, 1998, 79, 1725-1734; Khromykh and Westaway, *J. Virol.*, 1997, 71, 1497-1505; Molenkamp et al., *J. Virol.*, 2003, 77, 1644-1648; Shi et al., *Virology*, 2002, 296, 219-233; Varnavski and Khromykh, *Virology*, 1999, 255, 366-375; Perri et al., *J. Virol.*, 2000, 74, 9802-9807; Curtis et al., *J. Virol.*, 2002, 76, 1422-1434), the present inventors could also engineer a variety of JEV viral replicon vector RNAs that can be packaged when the structural proteins are supplied *in trans* by using the alphavirus-based expression system (Agapov et al., *Proc. Natl. Acad. Sci. USA*, 1998, 95, 12989-12994). Thus, the ability of packaging systems to efficiently generate biosafe JEV vectors has clearly been demonstrated. Unlike alphaviruses (Frolova et al., *J. Virol.*, 1997, 71, 248-258; White et al., *J. Virol.*, 1998, 72, 4320-4326) and retroviruses (Rein, *Arch. Virol. Suppl.*, 1994, 9, 513-522), little is known about the packaging signals employed by flaviviruses, including JEV. Our *trans*-complementation system for JEV provides evidence that suggests the whole JEV structural region is unlikely to

play a role in packaging. Thus, this system will be useful in defining the packaging signals in JEV RNA and the regions in the structural proteins that are involved in RNA encapsidation and morphogenesis. This information will further enhance the utility of our JEV-based expression systems.

In summary, the full-length JEV genomic RNA and the infectious JEV cDNA therefrom of the present invention are not only able to identify neurovirulence- and pathogenesis-related JEV genes but also available for the study of molecular mechanisms of JEV replication, transcription and translation. In addition, the full-length JEV genomic RNA and the infectious JEV cDNA can be effectively used for the development of treatment agents, vaccines, diagnostic reagents and diagnostic kits for JEV, and an expression vector for heterologous genes of interest in eukaryotic cells as well. Furthermore, the JEV-based vector system described in the present invention is a promising system by which foreign genes can be delivered into cells *in vitro* and possibly *in vivo* for DNA immunization and transient gene therapy.

BRIEF DESCRIPTION OF THE DRAWINGS

The application of the preferred embodiments of the present invention is best understood with reference to the accompanying drawings, wherein:

FIG. 1 is a set of photographs showing the comparison of large-plaque-forming JEV isolate CNU/LP2 and original K87P39 strain. (A-B) A set of photographs showing plaque morphology using BHK-21 cells (A) or Vero cells (B). BHK-21 (A) or Vero (B) cells were mock infected (Mock-infected) or infected with the original JEV K87P39 strain (K87P39-infected), which formed a heterogeneous mixture of viral plaque sizes. The CNU/LP2 isolate purified in the present invention formed a homogeneous population of large plaques (CNU/LP2-infected). (C) Levels and patterns of JEV protein expression. BHK-21 cells were mock infected or infected with K87P39, CNU/LP2 or the yellow fever virus strain YF17D. Eighteen hours later, they were fixed and stained with JEV-specific mouse hyperimmune ascites followed by fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (green fluorescence) and confocal microscopy. Nuclei were visualized by staining with propidium iodide (red fluorescence) in the presence of RNase A.

FIG. 2 is a set of diagrams and a pair of electrophoresis photographs showing strategies used to sequence genomic RNA of CNU/LP2. (A) A schematic diagram showing the RT-PCR amplification of three overlapping cDNA amplicons representing the entire JEV genomic RNA apart from the 5′ and 3′ termini. RNA is indicated in gray, and cDNA is indicated by solid parallel lines. The top panel schematically depicts the CNU/LP2 JEV genomic RNA (10,968 base pairs in length). The bottom panels portray the three overlapping cDNAs, JVF (nt 1 to 3865), JVM (nt 3266 to 8170), and JVR (nt 7565 to 10893). (B) A schematic diagram showing the procedure to sequence the 3′ end of CNU/LP2 genomic RNA. The 5′-phosphorylated and 3′-blocked oligonucleotide T (Oligo T) was ligated to the 3′ end of JEV genomic RNA by T4 RNA ligase, and the resulting RNA was then used for cDNA synthesis and amplification with the primers indicated by arrows. The resulting products were cloned and sequenced. (C) An electrophoresis photograph showing the JEV-specific amplicons synthesized from the oligonucleotide T-ligated JEV genomic RNA described in (B). First-strand cDNA was synthesized with oligonucleotide TR, complementary to oligonucleotide T, and the RT reaction

was carried out in the presence (lane 1) or absence (lane 2) of Superscript II reverse transcriptase. The cDNA was amplified with oligonucleotide TR and primer J35, which is complementary to nt 10259 to 10276. The expected size of the PCR product is 727 base pairs. The products were separated on a 1.2% agarose gel and visualized by staining with ethidium bromide (EtBr). (D) A schematic diagram showing the procedure to sequence the 5′ end of CNU/LP2 genomic RNA. The cap structure of viral genomic RNA was removed with tobacco acid pyrophosphatase, and the decapped viral RNA was then self-ligated with T4 RNA ligase and used for cDNA synthesis and amplification. The resulting amplified products were cloned and sequenced. (E) An electrophoresis photograph showing the JEV-specific amplicons synthesized from the self-ligated JEV genomic RNA described in (D). First strand cDNA synthesis was carried out with primer J40, which is complementary to nt 215 to 232. The RT reaction was performed in the presence (lane 1) or absence (lane 2) of Superscript II reverse transcriptase. The cDNA was amplified with primer J35 and primer J39, which is complementary to nt 164 to 181. The expected size of the PCR product is 890 base pairs. The amplified products were separated on a 1.2% agarose gel and visualized by staining with

EtBr. Lane M indicates a 100-bp DNA size ladder marker (in base pairs).

FIG. 3 is a set of diagrams showing the construction of full-length JEV cDNA clones in bacterial artificial chromosome (BAC) pBeloBAC11. (A) A schematic diagram of the full-length JEV cDNAs constructed in pBeloBAC11. Viral proteins are shown with thick solid lines at both termini representing the 5′ and 3′ NTRs of the viral genome. The SP6 and T7 promoter transcription start sites and the unique restriction endonuclease recognition site ensuring runoff transcription are shown at the 5′ and 3′ ends, respectively. (B-C) A set of schematic diagrams showing the 5′ and 3′ termini of full-length JEV cDNA clones. Nucleotide sequences of JEV genomic RNA are shown as bold italic lowercase letters. Illustrated are the 5′ termini of four SP6-driven (B) and four T7-driven (C) full-length JEV cDNA templates. To produce SP6 and T7 RNA polymerase runoff products, the 3′ termini of two SP6-driven (B, pBAC^{SP6}/JVFL/*Xho*I and pBAC^{SP6}/JVFLx/*Xho*I) and two T7-driven (C, pBAC^{T7}/JVFL/*Xho*I and pBAC^{T7}/JVFLx/*Xho*I) JEV cDNA templates were linearized by *Xho* I digestion, resulting in three nucleotides (CGA) of virus-unrelated sequence

at the 3′ ends. Similarly, the cutting of the 3′ termini of an SP6-driven (B, pBAC^{SP6}/JVFLx/*Xba*I) and a T7-driven (C, pBAC^{T7}/JVFLx/*Xba*I) JEV cDNA template with *Xba* I resulted in four nucleotides (CTAG) of virus-unrelated sequence at the 3′ ends. In contrast, the authentic 3′ end of JEV genomic RNA was present when SP6-driven (B, pBAC^{SP6}/JVFLx/*Xba*I^{MBN}) and T7-driven (C, pBAC^{T7}/JVFLx/*Xba*I^{MBN}) JEV cDNA templates were linearized by *Xba* I digestion and then treated with mung bean nuclease (MBN) to remove the unrelated single-stranded sequences. Underlined is the restriction endonuclease recognition site introduced at the 3′ end of the viral genome. An arrowhead indicates a cleavage site.

FIG. 4 is a set of a photograph and a graph showing the fact that full-length JEV cDNA template alone is not infectious but is required for the generation of infectious synthetic RNAs during *in vitro* transcription. (A) An electrophoresis photograph showing the cDNA template and synthetic RNA transcripts. (B) A graph showing infectivity obtained by transfecting BHK-21 cells with an *in vitro* transcription reaction mixture, which contains full-length JEV cDNA template and synthetic RNA transcripts. pBAC^{SP6}/JVFLx/*Xba*I (100-200 ng) linearized with *Xba* I

and treated with MBN was used for SP6 polymerase transcription in the absence (A, lane 1; B, Without Treatment) or presence (A, lane 2; B, Dnase I During) of DNase I. After synthesis, the transcription reaction mixture was treated for 30 min at 37°C with DNase I (A, lane 3; B, Dnase I After) or RNase A (A, lane 4; B, Rnase A After). As a control, the reaction was carried out in the absence of SP6 RNA polymerase (A, lane 5; B, Without SP6 Pol). (A) Following treatment, 5% of the reaction mixture was separated on a 0.6% agarose gel and the cDNA template and RNA transcripts were visualized by staining with EtBr. (B) The reaction mixtures were used to transfect BHK-21 cells, and infectious centers of plaques were estimated.

FIG. 5 is a set of photographs and graphs showing the comparison of synthetic JEVs with parental virus CNU/LP2. (A) Representative plaque assays of synthetic JEVs and parent CNU/LP2. BHK-21 cells were infected with parent or synthetic viruses, overlaid with agarose, and stained 3 days later with crystal violet. (B) Growth kinetics in BHK-21 cells of synthetic JEVs and parent CNU/LP2 infected at multiplicities of infection (MOI) of 0.01, 1, and 10. Viruses were harvested at the hour postinfection (h.p.i) indicated, and titers

were determined by plaque assays. (C-D) Viral protein and RNA levels were analyzed by immunoblotting (C) and Northern blotting (D), respectively. BHK-21 cells were infected at an MOI of 1 with synthetic JEVs (lanes 1-4) or CNU/LP2 (lane 5) or mock-infected (lane 6) and cultured for 18 hrs. (C) Protein extracts were prepared from approximately $3x10^4$ cells and separated on 10% SDS-polyacrylamide gels. Viral proteins were visualized by immunoblotting with JEV-specific mouse hyperimmune ascites (top panel). In parallel, actin protein was detected as a loading and transfer control (bottom panel). The positions of viral protein-related cleavage intermediates and actin are indicated with arrowheads on the left. Molecular mass markers in kDa are indicated on the right. (D) Total RNA from approximately $1x10^5$ cells was extracted and analyzed by Northern blotting using a ^{32}P-labeled antisense riboprobe hybridizing to the sequence in the NS5 gene encompassing nt 9143-9351 (top panel). Etbr-stained 18S rRNA bands are shown as a loading control (bottom panel). Full-length genomic viral RNA (11kb) and 18S rRNA are indicated on the left.

FIG. 6 is a set of diagrams and an electrophoresis photograph showing the presence of *Xho*

I genetic marker in recombinant JEVs derived from pBAC^{SP6}/JVFLx/gm/*Xba*I. (A) Schematic diagram of the RT-PCR fragments of JVFLx/*Xba*I^{MBN} and JVFLx/gm/*Xba*I^{MBN} expected after *Xho* I digestion. Indicated are the primers used for RT-PCR (arrows), the introduced *Xho* I site (asterisk), and the sizes of the RT-PCR products (2,580 bp) and the two *Xho* I digestion products (1,506 bp and 1,074 bp) expected after digestion of JVFLx/gm/*Xba*I^{MBN} with *Xho* I. (B) BHK-21 cells were transfected with synthetic RNAs transcribed from either pBAC^{SP6}/JVFLx/*Xba*I^{MBN} or pBAC^{SP6}/JVFLx/gm/*Xba*I^{MBN}. Viruses were recovered 24 hr later and serially passaged in BHK-21 cells at a multiplicity of infection of 0.1. At each passage prior to the next round of infection, viruses were incubated with DNase I and RNase A. At passage 1 and 3, viral RNA was extracted from the culture supernatant containing the released viruses and used for RT-PCR. The PCR products were incubated in the presence (+) or absence (-) of *Xho* I, separated on a 1% agarose gel, and stained with EtBr. The expected sizes of the undigested and digested PCR products are shown on the left. Lane M indicates a 1-kb DNA ladder marker (in base pairs).

FIG. 7 is a graph showing the specific infectivity of

synthetic RNAs transcribed from infectious JEV cDNA clones ($pBAC^{SP6}$/JVFLx/*Xba*I) propagated for 180 generations. Two independent clones carrying $pBAC^{SP6}$/JVFLx/*Xba*I (solid and open circles) were cultivated at 37°C overnight in 2xYT with chloramphenicol. The primary cultures were propagated every day for nine days by 10^6-fold dilution and adding fresh broth for overnight growth. Each passage was estimated to be about 20 generations. At the indicated passages, the DNA plasmids were purified, linearized by *Xba* I digestion and treated with MBN, and used as templates for runoff transcription using SP6 RNA polymerase. The transcripts were then used to transfect BHK-21 cells to determine specific infectivity.

FIG. 8 is a set of diagrams, photographs, and a graph showing the expression of foreign genes with JEV cDNA as a vector. (A) Schematic diagram of the cDNA templates used for runoff transcription with SP6 RNA polymerase. Indicated are the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES)-driven GFP or LUC genes that were inserted at the beginning of the 3'NTR of the viral genome, the SP6 promoter transcription start, and the runoff site generated by

Xba I digestion and MBN treatment (*Xba*I/MBN). In pBAC^{SP6}/JVFLx/LUC^{REP-}/*Xba*I^{MBN}, a solid vertical bar indicates an 83-nucleotide deletion (nt 5580-5663) in the middle of the NS3 gene that preterminates viral translation at nt 5596 (asterisk). (B) Expression of GFP protein. BHK-21 cells were mock-transfected (Mock) or transfected with 2 μg of synthetic RNAs transcribed from the pBAC^{SP6}/JVFLx/GFP/*Xba*I^{MBN} template (JVFLx/GFP/*Xba*I^{MBN}), incubated for 30 hr, and then fixed and examined by confocal microscopy. (C) Induction of LUC protein. BHK-21 cells ($8x10^6$) were mock-transfected or transfected with 2 μg of synthetic RNAs transcribed from the pBAC^{SP6}/JVFLx/LUC/*Xba*I^{MBN} (•) or pBAC^{SP6}/JVFLx/LUC^{REP-}/*Xba*I^{MBN} (o) templates, and seeded in a 6-well plate at a density of $6x10^5$ cells per well. Cells were lysed at the indicated time points and LUC activity was determined. The standard deviations obtained from three independent experiments are indicated by error bars.

Fig. 9 shows the construction and characterization of heterologous gene-encoding infectious recombinant JEVs that are based on the bicistronic full-length infectious JEV cDNA that serves as a BAC. (A) Strategy to construct the infectious

recombinant JEV cDNAs. The structure of the parental infectious JEV cDNA (pJEV/FL) is shown (Yun et al., *J. Virol.*, 2003, 77, 6450-6465). The viral ORFs are illustrated by thick solid lines at both termini that indicate the 5′ and 3′ NTRs of the viral genome. The additional expression unit driven by the EMCV IRES was inserted at the beginning of the 3′NTR using the unique natural *Nsi* I site. Indicated are the SP6 promoter transcription start site (SP6 promoter) and the runoff site generated by *Xba* I digestion and MBN treatment (*Xba*I/MBN). X indicates a foreign gene of interest. (B) The structures of the infectious recombinant JEV cDNAs constructed in the present invention are shown. Three commonly used reporters (EGFP, 768 bp; LUC, 1653 bp; and LacZ, 3012 bp) or a dominant selective marker PAC (600 bp) were engineered to be at the beginning of the 3′NTR. In case of the replication-competent pJEV/FL/LUC cDNA, the replication-incompetent pJEV/FL/LUC^{REP-} cDNA was also used as a negative control by introducing an 83-nucleotide deletion (■) in the middle of the NS3 gene, which results in the premature termination of viral translation at nt 5596 (*) as previously described (Yun et al., *J. Virol.*, 2003, 77, 6450-6465). (C-D) Comparison of the infectiousness of the recombinant JEVs with that of the parent. BHK-21

cells ($8X10^6$) were mock-transfected or transfected with 2 μg of the parent or recombinant JEV RNAs that had been transcribed from the relevant JEV cDNA, as indicated. (C) Representative plaques. The transfected cells were overlaid with agarose and stained 5 days later with crystal violet. (D) Viral protein accumulation. The transfected cells ($4X10^5$) were lysed with 1X sample loading buffer at the indicated time points and the protein extracts were resolved on 10% SDS-polyacrylamide gels. The viral proteins were visualized by immunoblotting with JEV-specific mouse hyperimmune sera (Yun et al., *J. Virol.*, 2003, 77, 6450-6465). The positions of the viral proteins (E and NS1) and the cleavage-related intermediates are indicated by arrowheads on the left. Molecular mass markers in kDa are indicated on the right. V indicates JEV CNU/LP2-infected BHK-21 cells and N indicates naïve BHK-21 cells.

Fig. 10 shows the expression of the commonly used reporter genes and a dominant selective marker using infectious JEV cDNA as the vector. BHK-21 cells ($8X10^6$) were mock-transfected or transfected with 2 μg of the parent or recombinant JEV RNAs that had been transcribed from each plasmid. (A-B) pJEV/FL/EGFP, (C)

pJEV/FL/LacZ, (D) pJEV/FL/LUC or pJEV/FL/LUC^{REP-}, (E) pJEV/FL/PAC. (A-B) Expression of EGFP. The transfected cells were prepared 36 hr posttransfection for confocal microscopy (A) and flow cytometric analysis (B). – indicates JEV/FL/EGFP RNA-transfected cells and ..... indicates mock-transfected cells. (C) Expression of LacZ. The transfected cells were processed for X-gal staining 36 hr posttransfection. (D) Induction of LUC. The transfected cells were seeded on six-well plates at a density of $4X10^5$ cells per well. At the indicated time points, the cell lysates were subjected to LUC assays. The experiments were done in triplicate and the mean values are shown by error bars. • indicates JEV/FL/LUC RNA-transfected cells, ○ indicates JEV/FL/LUC^{REP-} RNA-transfected cells, and – indicates the level of background luminescence of naïve cells. (E) Expression of PAC. The transfected cells were plated on a 6-well plate and incubated in complete media (dishes 1, 3, 5, and 7) or under a 0.5% agarose-containing overlay (dishes 2, 4, 6, and 8). After 2 days incubation, the plates were incubated for an additional 3 days in the presence of 10 μg/ml puromycin (dishes 5-8) or in its absence (dishes 1-4). The cells were then fixed and stained with crystal violet.

Fig. 11 shows the construction and vector characteristics of JEV viral replicons. (A) The structures of the JEV viral replicons are shown. Solid boxes (■) indicate in-frame deletions that had been introduced into the genome of the infectious pJEV/FL/LUC construct. Four constructs, namely, pJEV/Rep/ΔCC/LUC, pJEV/Rep/ΔC/LUC, pJEV/Rep/ΔprM/LUC, and pJEV/Rep/ΔE/LUC, contain a single in-frame deletion in each structural gene of JEV. pJEV/Rep/ΔCC/LUC has a deletion that extends to the proposed cyclization sequence motif in the 5′ region of the C gene, unlike pJEV/Rep/ΔC/LUC. Three constructs, namely, pJEV/Rep/ΔC+ΔprM/LUC, pJEV/Rep/ΔC+ΔE/LUC, and pJEV/Rep/ΔprM+ΔE/LUC, contain double in-frame deletions, while pJEV/Rep/ΔC+ΔprM+ΔE/LUC bears triple in-frame deletions in all of the structural proteins. Also engineered was pJEV/Rep/NS1/LUC, which encodes the 35 N-terminal and 24 C-terminal amino acids of the C protein followed immediately by the N-terminus of the NS1 protein and the rest of the viral genome. (B) Induction of LUC. Naïve BHK-21 cells ($8X10^6$) were transfected with 2 μg of the parent or JEV viral replicon RNAs that had been transcribed from each plasmid and then seeded on 6-well plates at a density

of $4X10^5$ cells per well. At the indicated time points, the cell lysates were subjected to LUC assays. The experiments were performed in triplicate and the mean values are shown. • black, pJEV/FL/LUC; ◆ black, pJEV/FL/LUC^{REP-}; ◆ blue, pJEV/Rep/ΔCC/LUC; ■ blue, pJEV/Rep/ΔC/LUC; ▲ blue, pJEV/Rep/ΔprM/LUC; • blue, pJEV/Rep/ΔE/LUC; ■ red, pJEV/Rep/ΔC+ΔprM/LUC; ▲ red, pJEV/Rep/ΔC+ΔE/LUC; • red, pJEV/Rep/ΔprM+ΔE/LUC; ■ green, pJEV/Rep/ΔC+ΔprM+ΔE/LUC; • green, pJEV/Rep/NS1/LUC. — indicates the level of background luminescence of naïve cells. (C) Viral protein accumulation. The transfected cells ($4X10^5$) were lysed with 1X sample loading buffer 48 hr posttransfection and the protein extracts were resolved on 10% SDS-polyacrylamide gels. The proteins were transferred onto the nitrocellulose membrane and immunoblotted with JEV-specific mouse hyperimmune sera.

Fig. 12 shows the construction of the packaging system for JEV viral replicons. (A) Structures of the JEV structural protein expression cassettes based on the Sindbis virus-based expression vector. pSinRep19 is the double subgenomic noncytopathic RNA vector. A foreign gene and the PAC gene are expressed by using separate subgenomic promoters, as indicated by arrows.

The pSinRep19/JEV C-E cassette encodes the JEV C, prM, and E genes. The pSinRep19/JEV C-E-*Bgl*II cassette encodes the JEV C, prM, and E genes, followed by the N terminal 58 residues of NS1, whereas the pSinRep19/JEV C-NS1 bears a remnant of the NS1 gene. MCS indicates multiple cloning sites. (B) Western blot analysis of the JEV structural proteins expressed from three JEV structural protein expression cassettes. The BHK-21 cells were mock-transfected or transfected with each JEV structural protein expression vector RNA and lysates were obtained 48 hr later. Equivalent amounts of cell lysates were resolved by SDS-PAGE and probed with the JEV-specific hyperimmune sera. Indicated are the positions of viral proteins E and NS1 on the right and the molecular mass markers in kDa on the left. (C) Schematic representation showing how JEV VRPs can be generated by (*i*) co-transfection of the JEV structural protein expression vector RNAs with JEV viral replicon RNAs or (*ii*) transfection of the JEV structural protein-expressing PCLs with JEV viral replicon RNAs. (D-E) The production of JEV VRPs. Two approaches were taken. One approach is involved the cotransfection of naïve BHK-21 cells with two vector RNAs, namely, JEV structural protein expression vector RNA and the JEV viral replicon vector RNA indicated (D). The other

approach involved JEV PCLs, which were transfected with the JEV viral replicon vector RNA indicated (E). The JEV viral replicon RNAs used were as follows: □ green, JEV/Rep/ΔC+ΔprM+ΔE/EGFP; ■ green, JEV/Rep/NS1/EGFP; □ blue, JEV/Rep/ΔC+ΔprM+ΔE/LacZ; ■ blue, JEV/Rep/NS1/LacZ; □ black, JEV/Rep/ΔC+ΔprM+ΔE/LUC; ■ black, JEV/Rep/NS1/LUC. The supernatants were collected 48 hr posttransfection and used to infect naïve BHK-21 cells for the titration of VRPs and the examination of the respective reporter gene expression. — indicates the level of background luminescence of naïve cells.

EXAMPLES

Practical and presently preferred embodiments of the present invention are illustrative as shown in the following Examples.

However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and improvements within the spirit and scope of the present invention.

Example 1: Isolation of JEV viruses

<1-1> Cell lines and viruses

BHK-21 cell line was provided from Dr. Charles M. Rice of the Rockefeller University, and maintained in alpha minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, vitamins, and antibiotics. All reagents used in cell culture were purchased from Gibco/BRL Life Technologies, Inc., Gaithersburg, MD. The Korean JEV strain K87P39 (Chung et al., *Am. J. Trop. Med. Hyg.*, 1996, 55, 91-97) was obtained from the Korean National Institute of Health. This JEV K87P39 was isolated from wild mosquitoes in Korea in 1987 and underwent five passages in suckling mouse brains. The YF17D yellow fever virus strain was generated from the infectious cDNA pACNR/YF17D (provided from Dr. Charles M. Rice) by SP6 polymerase runoff transcription as described bellow.

<1-2> Plaque purification

Cells infected with the JEV K87P39 strain were overlaid with MEM containing 10% fetal bovine serum and 0.5% SeaKem LE agarose (FMC BioProducts, Rockland, Maine) and incubated in a 5% CO_2, 37℃ incubator for 3 to 4 days. After being cultured for 3 to 4 days, the

infected cells were fixed with 3.7% formaldehyde at room temperature for 4 hr. Then, agarose covering the cells was removed. Plaques were visualized by crystal violet staining. As a result, K87P39 strain formed a heterogeneous mixture of viral plaque sizes (FIG. 1A, K87P39-infected).

Consequently, the present inventors performed the plaque purification assay with BHK-21 cells to isolate a homogeneous population of a large-plaque-forming variant that the present inventors named CNU/LP2. BHK-21 cells infected with the JEV K87P39 strain were overlaid with MEM containing 10% fetal bovine serum and 0.5% SeaKem LE agarose and incubated in a 5% CO_2, 37℃ incubator for 3 to 4 days. Individual plaques were picked with sterile Pasteur pipettes and resuspended in 1 ㎖ of MEM. Viruses were eluted from the agarose at 4℃ for 2 hr. The eluate was amplified only once in BHK-21 cells and stored at -80℃.

Plaque assay was performed to compare the viral plaque sizes of susceptible BHK-21 cells infected with JEV K87P39 and JEV CNU/LP2 strains. As a result, the viral plaque sizes of susceptible BHK-21 cells infected with K87P39 varied (FIG. 1A, K87P39-infected). On the other hand, the CNU/LP2 purified in the present invention formed a homogeneous population of large

plaques (FIG. 1A, CNU/LP2-infected). In addition, similar plaque morphologies were also observed when Vero cells were infected with JEV K87P39 and JEV CNU/LP2 strains (FIG. 1B).

<1-3> Immunofluorescence

In order to examine JEV expression in infected BHK-21 cells by confocal microscopy, cells ($2X10^5$) were seeded in a four-well chamber slide, incubated for 12 hr, and then mock-infected or infected at an MOI of 1 for 18 hr with either the original JEV K87P39 strain, the JEV CNU/LP2 isolate, or the YF17D strain. Immunostaining for JEV viral proteins was accomplished by first fixing the cells by incubation in phosphate-buffered saline (PBS) containing 0.37%(v/v) formaldehyde for 30 min at 25℃. The cells were then washed three times with PBS and permeabilized for 10 min at 37℃ with PBS containing 0.2%(v/v) Triton X-100. Thereafter, the cells were washed four times with PBS, rehydrated in PBS for 15 min, and blocked for 1 hr at 37℃ with PBS containing 5%(w/v) bovine serum albumin (BSA). The cells were then incubated for 2 hr at 25℃ with 1:500-diluted mouse hyperimmune ascites fluid specific for JEV, washed three times with PBS, incubated for 2 hr at 25℃ with 1:500-diluted FITC-

conjugated goat anti-mouse IgG (Jackson ImmunoResearch Labs Inc.), and washed again three times with PBS. Thereafter, the cells were incubated for 30 min at 37℃ in PBS containing 5 μg/㎖ of propidium iodide and 5 μg/㎖ of RNase A to localize the nuclei and mounted with 0.2 ml of 80% glycerol. Images were acquired on a Zeiss Axioskop confocal microscope equipped with a 63X objective with a Bio-Rad MRC 1024 and LaserSharp software.

Confocal microscopy with anti-JEV hyperimmune ascites revealed that CNU/LP2-infected BHK-21 cells expressed JEV viral proteins around the perinuclear membranes (FIG. 1C, CNU/LP2-infected), similar to K87P39-infected cells (FIG. 1C, K87P39-infected). This fluorescence staining was not observed in mock-infected BHK-21 cells (FIG. 1C, Mock-infected). As a negative control, BHK-21 cells infected with yellow fever virus 17D, a flavivirus closely related to JEV, did not stain with anti-JEV hyperimmune ascites (FIG. 1C, YF17D-infected). CNU/LP2 infection of a variety of animal cell lines, including the neuronal SHSY-5Y(human) and B103(mouse) cell lines and the nonneuronal Vero(monkey) and MDCK (dog) cell lines, resulted in high virus titers (10^6-10^7 PFU/㎖) in the culture supernatants. Thus, the present inventors decided to use CNU/LP2 as

the parental strain for developing a reverse genetics system for JEV.

Example 2: Complete nucleotide sequence analysis of JEV CNU/LP2 genomic RNA

Viral genomic RNA was extracted from 100 μℓ of virus-containing culture fluid with 300 μℓ of TRIzol LS reagent as recommended by the manufacturer (Gibco/BRL) and then resuspended in 20 μℓ of RNase-free water. To analyze the complete nucleotide sequence of the viral genomic RNA, five overlapping cDNAs (JVF, JVM, JVR, JV3NTR, and JV35NTR) representing the entire viral RNA genome were amplified by long RT-PCR (FIG. 2). Oligonucleotides used for cDNA synthesis and amplification were designed according to the consensus sequence of all 16 fully sequenced JEV RNA genomes available from the GenBank database (CH2195LA, CH2195SA, FU, GP78, HVI, JaGAr01, JaOArS982, K94P05, Vellore P20778, p3, SA(A), SA(V), SA14, SA14-14-2, TC, and TL strains).

<2-1> Nucleotide sequence analysis of JEV CNU/LP2 genomic RNA

For JVF amplicons (nt 1-3865), primer J7,

represented by SEQ. ID. No 1 and complementary to nt 3986-4003 of the JEV genome, was used for cDNA synthesis (FIG. 2A). The primers for PCR amplification were primer J8 represented by SEQ. ID. No 2 and complementary to nt 1-18, and primer J6 represented by SEQ. ID. No 3 and complementary to nt 3845-3865. For JVM amplicons (nt 3266-8170), primer J4, represented by SEQ. ID. No 4 and complementary to nt 8150-8170 of the JEV genome, was used for cDNA synthesis. The primers for PCR amplification were primer J20 represented by SEQ. ID. No 5 and complementary to nt 3266-3283, and primer J4. For JVR amplicons (nt 7565-10893), primer J1, represented by SEQ. ID. No 6 and complementary to nt 10947-10967 of the JEV genome, was used for cDNA synthesis. The primers for PCR amplification were primer J12 represented by SEQ. ID. No 7 and complementary to nt 7565-7582, and primer J2 represented by SEQ. ID. No 8 and complementary to nt 10870-10893. The standard RT reaction was conducted in a 20-μℓ reaction mixture containing 10 μℓ of extracted viral RNA, 5 p mol of the appropriate primer, 100 U of Superscript II reverse transcriptase (Gibco/BRL), 40 U of RNaseOUT (Gibco/BRL), 0.1 mM dithiothreitol (DTT), 10 mM deoxynucleotide triphosphate (dNTP) mix, and the RT buffer supplied by the manufacturer (Gibco/BRL).

The reaction mixture was incubated at 37℃ for 1 hr and then heated at 70℃ for 15 min. A 5-㎕ aliquot of the RT mixture was subsequently used for PCR amplification with Pyrobest DNA polymerase (Takara Bio Inc., Shiga, Japan) and the appropriate primer pair. The PCRs were performed with 30 cycles of denaturation at 94℃ for 30 sec, annealing at 60℃ for 30 sec, and extension at 72℃ for 5 min, followed by a final extension at 72℃ for 10 min. To avoid the selection bias that can occur due to cloning, the uncloned materials of the amplified products were directly sequenced in both directions with an automatic 3700 DNA sequencer. Sequencing analysis with two independently isolated preparations of viral RNA resulted in identical sequences.

As a result, the complete nucleotide sequence of the entire viral genome of JEV CNU/LP2 except for 3′- and 5′-terminal regions was determined and represented by SEQ. ID. No 9.

<2-2> Determination of 3′-terminal sequence of JEV CNU/LP2 genomic RNA

In order to sequence the 3′-terminal sequences of the JEV CNU/LP2 genomic RNA, a synthetic oligonucleotide T represented by SEQ. ID. No 10 was ligated to the 3′ end of the viral genomic RNA to

provide a primer-binding site for cDNA synthesis and PCR amplification (Kolykhalov et al., *J. Virol.*, 1996, 70, 3363-3371). The 3′ end of oligonucleotide T was first modified by incorporating ddATP with terminal deoxynucleotidyltransferase (Takara), which blocks the intramolecular and intermolecular ligation of oligonucleotide T. The 5′ end of oligonucleotide T was also phosphorylated with T4 polynucleotide kinase (Takara). Thereafter, the modified oligonucleotide T was ligated to the 3′ end of the viral genomic RNA by T4 RNA ligase (New England Biolabs, Inc., Beverly, MA). The 20 μℓ of ligation reaction mixture contained 10 U of T4 RNA ligase, 40 U of RNaseOUT, 10 p mol of oligonucleotide T, viral genomic RNA, and the buffer supplied by the manufacturer (NEB). After incubation at 16℃ for 12 hr, the ligated viral RNA was phenol extracted, precipitated with ethanol, and resuspended with 20 μℓ of RNase-free water. Subsequently, 10 μℓ of the oligonucleotide-ligated viral RNA was used for cDNA synthesis with oligonucleotide TR represented by SEQ. ID. No 11, which is complementary to oligonucleotide T, as previously described. First-strand cDNA was amplified with primer J35 represented by SEQ. ID. No 12 and complementary to nt 10259 to 10276, and primer TR. For PCR, 5 μℓ aliquot of the RT reaction mixture was

amplified with Pyrobest DNA polymerase and 30 cycles of 30 sec at 94℃, 30 sec at 60℃, and 1 min at 72℃, followed by a final extension of 10 min at 72℃. The PCR mixtures were as described above. The cDNA amplicons designated as JV3NTR were cloned into the pRS2 vector (provided by Dr. Charles M. Rice) with *Hin*d III and *Eco*R I sites incorporated in the positive-sense and negative-sense primers, respectively (FIG. 2B).

As a result of agarose gel electrophoresis, it was revealed that the amplified products migrated as two bands, a larger band of approximately 700 bp and a smaller band of about 450 bp (FIG. 2C). Both bands were purified and cloned, and 20 and 10 randomly picked clones containing the larger and the smaller bands, respectively, were sequenced. As has been documented for most of the fully sequenced JEV isolates, the present inventors found that all the clones with the larger insert (about 700 bp) terminated the viral genome with -GATCT^{10968}. In contrast, all the clones with the smaller insert (about 450 bp) showed the viral genome truncated at nt 10684, resulting in a band 284 bp shorter. During assembly of the full-length JEV cDNA, the present inventors used the nucleotide sequences of the larger insert because the smaller insert did not contain 284 nucleotides at the 3′ end of

the viral genome.

<2-3> Determination of 5'-terminal sequence of JEV CNU/LP2 genomic RNA

The 5'-terminal sequence of JEV CNU/LP2 genomic RNA was determined by self-ligation of viral RNA (Campbell and Pletnev, *Virology*, 2000, 269, 225-237). The cap structure of viral genomic RNA was first cleaved off with tobacco acid pyrophosphatase (TAP). The cleavage reaction mixture (20 μℓ) contained 10 U of TAP (Epicentre Technology Co., Madison, WI), 10 μℓ of viral RNA, and the buffer supplied by the manufacturer (Epicentre Technology Co.). After incubation at 37℃ for 1 hr, the TAP-treated viral RNA was subjected to phenol extraction and ethanol precipitation, and resuspended with 20 μℓ of RNase-free water. Half (10 μℓ) of the decapped viral RNA was self-ligated in a 20-μℓ reaction mixture with T4 RNA ligase as described above. A quarter (5 μℓ) of the self-ligated viral RNA was used for cDNA synthesis with primer J40, represented by SEQ. ID. No 13 and complementary to nt 215 to 232. First-strand cDNA was PCR amplified with primer J39 represented by SEQ. ID. No 14 and complementary to nt 164 to 181, and primer J35 (FIG. 2D). Agarose gel electrophoresis revealed the

amplified products as a single band of about 850 bp (FIG. 2E). The amplified cDNA amplicons (JV35NTR) were digested with *Apo* I and *Spe* I, and ligated into the pRS2 vector which had been digested with *Apo* I and *Xba* I, leading to construct pRS2/JV3'5'.

To sequence the 5'-terminal sequences of the JEV CNU/LP2 genomic RNA, 12 randomly picked clones were sequenced. In all 12 clones, the present inventors found that the -GATCT^{10968} of the viral 3'-terminal sequence was followed by the 5'-terminal sequence ^{1}AGAAGT- (FIG. 2B and 2C). Identical results were also obtained by direct cycle sequencing of uncloned material. Thus, the present inventors have determined the complete nucleotide sequence of the CNU/LP2 isolate and confirmed that the sequence is represented by SEQ. ID. No 15.

Example 3: Construction of full-length infectious cDNAs for JEV

During our initial attempts to clone the cDNA of the CNU/LP2 RNA genome, it became apparent that a particular region of the viral genome was not compatible with cloning in high-copy-number plasmids in *E. coli* because the cloned DNA underwent genetic

rearrangements. These difficulties have also been reported for other flaviviruses (Campbell and Pletnev, *Virology*, 2000, 269, 225-237; Polo et al., *J. Virol.*, 1997, 71, 5366-5374; Gritsun and Gould, *Virology*, 1995, 214, 611-618; Sumiyoshi et al., *J. Infect. Dis.*, 1995, 171, 1144-1151; Sumiyoshi et al., *J. Virol.*, 1992, 66, 5425-5431; Rice et al., *New Biol.*, 1989, 1, 285-296). Attempts to clone this region into a low-copy-number bacterial plasmid were also unsuccessful due to genetic instability together with a low DNA yield. Thus, the present inventors used the bacterial artificial chromosome (BAC) plasmid pBeloBAC11 as a vector to house full-length infectious cDNAs for JEV.

<3-1> Subcloning of three long overlapping JEV cDNA amplicons

The present inventors used recombinant DNA techniques according to standard procedures (Sambrook et al., *Molecular cloning*, 1989, Cold Spring Harbor Laboratory). First, three overlapping cDNA amplicons (JVF, JVM and JVR) originally used for complete nucleotide sequence analysis were subcloned into pBAC/SV represented by SEQ. ID. No 42, a derivative of the pBeloBAC11 plasmid. The pBAC/SV plasmid contains the 491-bp *Not* I-*Aat* II (T4 DNA polymerase-treated)

fragment of pACNR/NADL (Mendez et al., *J. Virol.*, 1998, 72, 4737-4745), the 9,215-bp *Sac* I (T4 DNA polymeras-treated)-*Ssp* I (T4 DNA polymerase-treated) fragment of pSINrep19 (Frolov et al., *Proc. Natl. Acad. Sci., USA.*, 1996, 93, 11371-11377), and the 6,875-bp *Sfi* I (T4 DNA polymerase-treated)-*Not* I fragment of pBeloBAC11. Thus, the 3,863-bp *Rsr* II -*Avr* II fragment of the JVF amplicons, the 4,717-bp *Bsp*E I-*Mlu* I fragment of the JVM amplicons, and the 3,326-bp *Rsr* II-*Bgl* II fragment of the JVR amplicons were inserted into the pBAC/SV plasmid, which had been digested with the same enzymes. This led to the pBAC/JVF, pBAC/JVM, and pBAC/JVR subclone constructs, respectively. These BAC plasmids were grown in *E. coli* DH10B cells and sequenced. The nucleotide sequences of the cloned cDNAs were identical to that of CNU/LP2 with the exception of a point mutation, $T^{8906} \rightarrow C$ (silent), within the NS5 gene in pBAC/JVR. The $T^{8906} \rightarrow C$ substitution was translationally silent and must have arisen during the cloning because sequencing of eight randomly picked individual clones revealed a T residue at nt 8906. Although the $T^{8906} \rightarrow C$ substitution does not alter the corresponding amino acid, it is possible that this change could affect viral replication (van Dinten et al., *Proc. Natl. Acad. Sci. USA*, 1997, 94, 991-996),

and thus the present inventors corrected this substitution back to a T residue. The $T^{8906} \rightarrow C$ substitution was corrected by recloning a 315-bp *Apa* I-*Hin*d III fragment corresponding to nt 8827 to 9142, leading to the construct pBAC/JVRR. During their manipulation and propagation in the *E.coli* strain DH10B, all three subcloned JEV cDNAs remained genetically stable.

<3-2> Insertion of SP6 promoter into the 5′ end of the full-length JEV cDNA

In order to facilitate the precise adjoining of the bacteriophage SP6 promoter transcription start to the 5′ end of the full-length JEV cDNA, the present inventors modified the pBAC/JVF. First, two fragments were isolated by PCR of pBAC/SV with primer J41 represented by SEQ. ID. No 16 and primer J43 represented by SEQ. ID. No 17, which incorporates the negative-sense sequence of the SP6 promoter and PCR of pBAC/JVF with primer J42 represented by SEQ. ID. No 18 and primer J40 represented by SEQ. ID. No 19. These two fragments were fused by a second round of PCR with primers J41 and J40. The resulting amplicons were digested with *Pac* I and *Pme* I, and ligated with pBAC/JVF which had been digested with the same two

enzymes. This produced pBAC^{SP6}/JVF.

<3-3> Construction of full-length JEV cDNAs containing SP6 promoter

In order to generate an authentic or nearly authentic 3′ terminus during runoff transcription of plasmid linearized at the 3′ end of the viral genome, the present inventors modified pBAC/JVRR so that the nucleotide sequence of the authentic 3′ terminus was followed by a unique restriction endonuclease recognition site, either *Xho* I or *Xba* I. To create the pBAC/JVRR/*Xho*I subclone containing a unique *Xho* I site at the end of the viral genome, fragment I was synthesized by PCR amplification of pRS2/JV3′5′ with primer J90 represented by SEQ. ID. No 20 and primer J45 represented by SEQ. ID. No 21, which incorporates an *Xho* I site. The 298-bp *Sfi* I-*Spe* I portion of fragment I amplicons was ligated with pBAC/JVRR which had been digested with *Sfi* I and *Nhe* I. To create pBAC/JVRRx/*Xba*I, which has an *Xba* I site at the end of the viral genome, the existing *Xba* I site at nt 9,131 to 9,136 within the NS5 gene was first inactivated by introducing a silent point mutation ($A^{9134} \rightarrow T$) by PCR. In this construct, the "x" denotes the presence of the silent point mutation ($A^{9134} \rightarrow T$) that destroyed the

original *Xba* I site. Particularly, pBAC/JVRR was amplified with primer J31 represented by SEQ. ID. No 22 and primer J47 represented by SEQ. ID. No 23, which incorporated the $A^{9134} \rightarrow T$ substitution. The 315-bp *Apa* I-*Hind* III portion of the cDNA amplicons, corresponding to nt 8,828 to 9,143, was cloned into pBAC/JVRR, leading to the construct pBAC/JVRRx. Subsequently, pBAC/JVRRx/*Xba*I was constructed in the same manner as described for pBAC/JVRR/*Xho*I. Thus, fragment II was obtained by PCR amplification of pRS2/JV3'5' with primer J90 and primer J46 represented by SEQ. ID. No 24, which incorporated an *Xba* I site. The 298-bp *Sfi* I-*Spe* I portion of the fragment II amplicons was then ligated into pBAC/JVRRx which had been digested with *Sfi* I and *Nhe* I. To create pBAC/JVRRx/*Xho*I containing a unique *Xho* I site and the $A^{9134} \rightarrow T$ substitution, the 298-bp *Sfi* I-*Spe* I portion of fragment I amplicons was ligated into pBAC/JVRRx which had been digested with *Sfi* I and *Nhe* I.

Thus, the present inventors constructed five plasmids, $pBAC^{SP6}$/JVF, pBAC/JVM, pBAC/JVRR/*Xho*I, pBAC/JVRRx/*Xba*I, and pBAC/JVRRx/*Xho*I. These plasmids contained contiguous regions of the JEV genome and could now be used to assemble three different full-length JEV cDNAs (FIG. 3). First, the $pBAC^{SP6}$/JVFM

subclone was constructed by ligating together the 4,717-bp *Bsp*E I-*Mlu* I fragment of pBAC/JVM, the 8,970-bp *Bsp*E I-*Xba* I fragment of pBAC^{SP6}/JVF, and the 3,670-bp *Xba* I-*Mlu* I fragment of pBAC/SV. Subsequently, two fragments of pBAC^{SP6}/JVFM (the 8,142-bp *Pac* I-*Sap* I fragment and the 4,801-bp *Pac* I-*Bsr*G I fragment) were ligated with either i) the 5,620-bp *Sap* I-*Bsr*G I fragment of pBAC/JVRR/*Xho*I to generate pBAC^{SP6}/JVFL/*Xho*I, ii) the 5,622-bp *Sap* I-*Bsr*G I fragment of pBAC/JVRRx/*Xba*I to generate pBAC^{SP6}/JVFLx/*Xba*I, or iii) the 5,620-bp *Sap* I-*Bsr*G I fragment of pBAC/JVRRx/*Xho*I to generate pBAC^{SP6}/JVFLx/*Xho*I. Finally, three assembled full-length JEV cDNAs were designated pBAC^{SP6}/JVFL/*Xho*I, pBAC^{SP6}/JVFLx/*Xho*I, and pBAC^{SP6}/JVFLx/*Xba*I and represented by SEQ. ID. No 43, No 44, and No 45, respectively (FIG. 3B). These cDNA clones all had the SP6 promoter transcription start at the beginning of the viral genome so that synthetic RNA transcripts with an authentic 5′ end would be generated through *in vitro* transcription using SP6 RNA polymerase (FIG. 3B, gray box). To ensure that the 3′ end of the viral genome after runoff transcription would be close to authentic, the present inventors placed a unique restriction endonuclease recognition site, either *Xho* I or *Xba* I, at the end of the viral genome (FIG. 3B,

underlined). Thus, pBAC^{SP6}/JVFL/*Xho*I bears an *Xho* I site at the end of the viral genome. For the construct with an *Xba* I site immediately at the end of viral genome, as the viral genome already contains an *Xba* I site in the NS5 gene, this site had to be destroyed by introducing a silent point mutation ($A^{9134} \rightarrow T$). This construct was designated pBAC^{SP6}/JVFLx/*Xba*I, where the "x" denotes the presence of the silent point mutation that destroyed the original *Xba* I site. The third clone, pBAC^{SP6}/JVFLx/*Xho*I, contains both the *Xho* I site at the end of viral genome and the $A^{9134} \rightarrow T$ substitution.

The present inventors deposited the pBAC^{SP6}/JVFLx/*Xba*I at Gene Bank of Korea Research Institute of Bioscience and Biotechnology (KRIBB) on October 2, 2002 (Accession No: KCTC 10347BP).

<3-4> Construction of full-length JEV cDNAs containing T7 promoter

In addition to the SP6-driven JEV cDNAs, the present inventors also constructed a set of three T7-driven full-length JEV cDNAs in a similar manner of the Example <3-3>. First, a fragment from pBAC/NADLcIn-/PAC (provided by Dr. Charles M. Rice) was synthesized by PCR with the primer J81 represented by SEQ. ID. No

25 and the primer J80 represented by SEQ. ID. No 26. A fragment from pBAC^{SP6}/JVFLx/*Xba*I was also synthesized with the primer J42 represented by SEQ. ID. No 27 and the primer J82 represented by SEQ. ID. No 28. These two fragments were fused by the second round of PCR with the primers J81 and J82. The 793-bp *Eco*R I-*Spe* I fragment of the resulting amplicons was inserted into the pRS2 vector digested with *Eco*R I and *Xba* I, leading to the construct pRS2^{T7}/5'JV. The 675-bp *Pvu* I-*Pme* I fragment of pRS2^{T7}/5'JV was ligated with either i) the 18,364-bp *Pac* I-*Pme* I fragment of pBAC^{SP6}/JVFL/*Xho*I to create pBAC^{T7}/JVFL/*Xho*I, ii) the 18,364-bp *Pac* I-*Pme* I fragment of pBAC^{SP6}/JVFLx/*Xho*I to create pBAC^{T7}/JVFLx/*Xho*I, or iii) 18,366-bp *Pac* I-*Pme* I of pBAC^{SP6}/JVFLx/*Xba*I to create pBAC^{T7}/JVFLx/*Xba*I. Finally, three assembled full-length JEV cDNAs were designated pBAC^{T7}/JVFL/*Xho*I, pBAC^{T7}/JVFLx/*Xho*I, and pBAC^{T7}/JVFLx/*Xba*I and represented by SEQ. ID. No 46, No 47, and No 48, respectively (FIG. 3C). At every cloning step during the assembly process, the structural integrity of the cloned cDNAs was assessed by extensive restriction and nucleotide sequence analyses. Structural instability of the inserts leading to deletions or rearrangements was not observed.

The present inventors deposited the

pBAC^{T7}/JVFLx/*Xba*I at Gene Bank of Korea Research Institute of Bioscience and Biotechnology (KRIBB) on October 2, 2002 (Accession No: KCTC 10346BP).

Example 4: Transcriptions and transfections

The present inventors synthesized RNA transcripts by *in vitro* transcription. Particularly, 100 to 200 ng of the template DNA linearized with *Xho* I or *Xba* I digestion and in some cases modified with MBN was added to a 25-μℓ reaction mixture consisting of the buffer supplied by the manufacturer (Gibco/BRL) plus 0.6 mM cap analog [m^7G(5')ppp(5')A or m^7G(5')ppp(5')G, NEB Inc.], 0.5 μM [^{3}H]UTP (1.0 mCi/mℓ, 50 Ci/m mol, New England Nuclear Corp., Boston, MA), 10 mM DTT, 1 mM each UTP, GTP, CTP and ATP, 40 U of RNaseOUT, and 15 U of SP6 RNA polymerase (Gibco/BRL). The reaction mixtures were incubated at 37℃ for 1 hr. RNAs were quantified on the basis of [^{3}H]UTP incorporation as measured by RNA adsorption to DE-81 (Whatman, Maidstone, UK) filter paper (Sambrook et al., *Molecular cloning*, 1989, Cold Spring Harbor Laboratory). A 1- to 1.5-μℓ aliquot of reaction mixture was examined by agarose gel electrophoresis, and aliquots were stored at -80℃ until use.

For RNA transfection, cells were electroporated with synthetic RNAs with a model ECM 830 electroporator (BTX Inc., San Diego, CA), as recommended by the manufacturer. Briefly, subconfluent cells were trypsinized, washed three times with ice-cold RNase-free PBS, and resuspended at a density of $2x10^7$ cells/ Ml in PBS. A 400-µl aliquot of the suspension was mixed with 2 µg of synthetic RNA, and the cells were immediately electroporated under the conditions determined previously to be optimal (980 V, 99-µs pulse length, and five pulses). The electroporated mixture was then transferred to 10 Ml of fresh medium.

An infectious center assay was used to quantify the specific infectivity of the synthetic RNA. Particularly, for runoff transcription, JEV cDNA templates were linearized by digestion with *Xho* I or *Xba* I. SP6 polymerase runoff transcription of the two *Xho* I-linearized plasmids ($pBAC^{SP6}$/JVFL/*Xho*I and $pBAC^{SP6}$/JVFLx/*Xho*I) in the presence of the $m^7G(5')ppp(5')A$ cap structure analog yielded capped synthetic RNAs containing three nucleotides (CGA) of virus-unrelated sequence at their 3′ ends (FIG. 3B). This is the result of copying the 5′ overhang left by the *Xho* I digestion (FIG. 3B). Similarly, SP6 polymerase runoff transcription of the *Xba* I-linearized

pBAC^{SP6}/JVFLx/*Xba*I plasmid in the presence of the $m^7G(5')ppp(5')A$ cap structure analog produced capped synthetic RNAs with four nucleotides (CTAG) of virus-unrelated sequence at their 3′ ends (FIG. 3B). The electroporated cells were serially diluted 10-fold and plated on monolayers of untransfected cells ($5x10^5$) in a six-well plate. Cells were allowed to attach to the plate for 6 hr, after which they were overlaid with 0.5% SeaKem LE agarose-containing MEM as described above. The plates were incubated for 3 to 4 days at 37℃ with 5% CO_2, and infectious plaque centers were visualized by crystal violet staining.

When susceptible BHK-21 cells were transfected with the synthetic RNAs from these constructs, all were highly infectious (Table 3). That is, the synthetic RNAs obtained from pBAC^{SP6}/JVFL/*Xho*I, pBAC^{SP6}/JVFLx/*Xho*I, and pBAC^{SP6}/JVFLx/*Xba*I transfected under optimal electroporation conditions had specific infectivities of $3.5x10^5$, $4.3x10^5$, and $3.4x10^5$ PFU/μg, respectively (Table 3, infectivity). Similar results were also obtained with synthetic RNAs transcribed from the T7-driven cDNA constructs by T7 polymerase runoff transcription (Table 3, infectivity).

<Table 3>

Specific infectivity of *in vitro* RNA transcripts generated from full-length JEV cDNAs and virus titer

Templates used for transcription[a]	Infectivity[b] (PFU/μg of RNA)	Virus titer[c] (PFU/Mℓ)	
		24 hr	48 hr
pBAC^{SP6}/JVFL/*Xho*I	3.5×10^5	4.4×10^5	3.6×10^6
pBAC^{T7}/JVFL/*Xho*I	2.9×10^5	2.0×10^5	2.3×10^6
pBAC^{SP6}/JVFLx/*Xho*I	4.3×10^5	2.1×10^5	5.2×10^6
pBAC^{T7}/JVFLx/*Xho*I	3.8×10^5	3.3×10^5	4.1×10^6
pBAC^{SP6}/JVFLx/*Xba*I	3.4×10^5	3.5×10^5	3.2×10^6
pBAC^{T7}/JVFLx/*Xba*I	3.0×10^5	2.4×10^5	2.7×10^6
pBAC^{SP6}/JVFLx/*Xba*I^{MBN}	3.1×10^6	6.2×10^6	1.4×10^6
pBAC^{T7}/JVFLx/*Xba*I^{MBN}	2.7×10^6	5.6×10^6	2.4×10^6

[a] : All full-length JEV cDNAs were linearized with an appropriate restriction endonuclease for runoff transcription as indicated in the names of the cDNAs. For pBAC^{SP6}/JVFLx/*Xba*I^{MBN} and pBAC^{T7}/JVFLx/*Xba*I^{MBN}, these cDNA templates were prepared by linearization with *Xba* I digestion, which was followed by treatment with MBN.

[b] : After *in vitro* transcription with SP6 or T7 RNA polymerase, as indicated, samples were used to electroporate BHK-21 cells, and infectious plaque centers were determined.

[c] : Virus titers at 24 and 48 hr postelectroporation.

<4-1> Construction of JEV RNA transcripts lacking the virus-unrelated sequences at their 3′ ends

It has been reported that for some flaviviruses, the presence of unrelated sequences at the 3′ end of synthetic RNAs transcribed from infectious cDNA diminishes or abrogates their specific infectivity (Yamshchikov et al., *Virology*, 2001, 281, 294-304). Based on this report, the present inventors generated synthetic RNAs lacking the virus-unrelated sequences at their 3′ends and compared their specific infectivities. Particularly, the present inventors generated synthetic JEV RNAs lacking the virus-unrelated sequences by treating the *Xba* I-linearized pBAC^{sp6}/JVFLx/*Xba*I plasmid with MBN prior to the transcription reaction, which removed the four excess nucleotides of CTAG. RNA transcripts from *Xba* I-linearized and MBN-treated pBAC^{SP6}/JVFLx/*Xba*I and pBAC^{T7}/JVFLx/*Xba*I (pBAC^{SP6}/JVFLx/*Xba*I^{MBN}, FIG. 3B and pBAC^{T7}/JVFLx/*Xba*I^{MBN}, FIG. 3C) both had increased specific infectivities compared to the untreated transcripts. Precisely, the specific infectivity of RNAs transcribed from pBAC^{SP6}/JVFLx/*Xba*I^{MBN} was estimated to be 3.1 x 10^6 PFU/μg, approximately 10-fold higher than the specific infectivity (3.4 x 10^5 PFU/μg) of the unmodified

template (Table 3, infectivity). The RNAs derived from pBAC^{T7}/JVFLx/*Xba*I also had increased specific infectivity after MBN modification (2.7 x 10^6 PFU/μg) (Table 3, infectivity). Therefore, the present inventors demonstrated that the authentic 3′ end of the JEV genome should be present to ensure highly infectious synthetic JEV RNA transcripts are generated.

In addition, the altered specific infectivity of the RNA transcripts due to the presence of three or four virus-unrelated nucleotides at the 3′ end also influences the virus titers harvested from culture supernatants of the transfected BHK-21 cells. Virus titers released from BHK-21 cells transfected with RNA transcripts from MBN-untreated pBAC^{SP6}/JVFL/*Xho*I, pBAC^{SP6}/JVFLx/*Xho*I, and pBAC^{SP6}/JVFLx/*Xba*I ranged from 2.1 x 10^5 to 4.4 x 10^5 PFU/Mℓ at 24 hr posttransfection (Table 3, virus titer 24 hr), at which time half of the transfected cells were still attached to culture dishes showing virus-induced strong cytopathic effect. These titers increased about 10-fold to the range of 3.2 x 10^6 to 5.2 x 10^6 PFU/Mℓ at 48 hr posttransfection (Table 3, virus titer 48 hr), at which point most of the cells had died and detached from the bottom of the culture dishes. In contrast, the virus titer released from BHK-21 cells transfected with RNA transcripts from MBN-

treated pBAC^{SP6}/JVFLx/*Xba*I^{MBN} had already reached 6.2 x 10^6 PFU/Mℓ at 24 hr posttransfection, at which time the majority of the transfected cells had died (Table 3, virus titer 24 hr). This titer decreased slightly to 1.4 x 10^6 PFU/Mℓ at 48 hr posttransfection (Table 3, virus titer 48 hr). Similar patterns of virus production were seen with the T7 polymerase-driven RNA transcripts (Table 3).

Example 5: Confirmation of specific infectivity of synthetic RNA transcripts

The present inventors confirmed that specific infectivity requires the transcription of RNA from the full-length JEV cDNA template by using the full-length cDNA clone pBAC^{SP6}/JVFLx/*Xba*I^{MBN} (FIG. 4). The cDNA template alone was not infectious (FIG. 4A, lane 5 and B, without SP6 Pol), but the intact cDNA template was needed during the transcription reaction because DNase I treatment abolished infectivity (FIG. 4A, lane 2 and B, DNase I During). Addition of DNase I after the transcription reaction had no effect (FIG. 4A, lane 3 and B, DNase I after) relative to the intact reaction mixture (FIG. 4A, lane 1 and B, without treatment), but RNase A treatment abolished the infectivity of the

transcribed synthetic RNAs (FIG. 4A, lane 4 and B, RNase A after).

Example 6: Comparison of synthetic JEVs recovered from full-length infectious cDNAs with the CNU/LP2 parental virus

The present inventors compared the synthetic JEVs recovered from full-length infectious cDNAs (pBAC^{SP6}/JVFL/*Xho*I, pBAC^{SP6}/JVFLx/*Xho*I, pBAC^{SP6}/JVFLx/*Xba*I, and pBAC^{SP6}/JVFLx/*Xba*I^{MBN}) with the parental virus CNU/LP2 originally used for cDNA construction (plaque morphology, growth kinetics, protein expression, RNA production, etc).

<6-1> Comparison of plaque morphology by plaque assay

BHK-21 cells were infected with the synthetic JEVs recovered from full-length infectious cDNAs (pBAC^{SP6}/JVFL/*Xho*I, pBAC^{SP6}/JVFLx/*Xho*I, pBAC^{SP6}/JVFLx/*Xba*I, and pBAC^{SP6}/JVFLx/*Xba*I^{MBN}) and the parental virus CNU/LP2. The cells were overlaid with MEM containing 10% fetal bovine serum and 0.5% SeaKem LE agarose (FMC BioProducts, Rockland, Maine) and incubated in a 5% CO_2, 37℃ incubator for 3 to 4 days. After being cultured

for 3 to 4 days, the infected cells were fixed with 3.7% formaldehyde at room temperature for 4 hr. Then, agarose covering the cells was removed. Plaques were visualized by crystal violet staining. As shown in FIG. 5A, BHK-21 cells infected with synthetic JEVs recovered from pBAC^{SP6}/JVFL/*Xho*I (dish 1), pBAC^{SP6}/JVFLx/*Xho*I (dish 2), pBAC^{SP6}/JVFLx/*Xba*I (dish 3), and pBAC^{SP6}/JVFLx/*Xba*I^{MBN} (dish 4) formed homogeneous large plaques, similar to the cells infected with CNU/LP2 (dish 5).

<6-2> Comparison of growth kinetics

The present inventors infected BHK-21 cells with the synthetic JEVs recovered from full-length infectious cDNAs (pBAC^{SP6}/JVFL/*Xho*I, pBAC^{SP6}/JVFLx/*Xho*I, pBAC^{SP6}/JVFLx/*Xba*I, and pBAC^{SP6}/JVFLx/*Xba*I^{MBN}) and the parental virus CNU/LP2. BHK-21 cells were infected with low (0.01 PFU/cell), medium (1.0 PFU/cell), and high (10 PFU/cell) MOI, after which the cell culture fluids were harvested periodically and used to determine the kinetics of infectious virus release over time. Particularly, viruses were harvested at the indicated time points, and titers were determined by plaque assay. As shown in FIG. 5B, the MOI-dependent virus titers accumulating over time were similar for the four recovered viruses (pBAC^{SP6}/JVFL/*Xho*I,

pBAC^{SP6}/JVFLx/*Xho*I, pBAC^{SP6}/JVFLx/*Xba*I, and pBAC^{SP6}/JVFLx/*Xba*I^{MBN}) and the parental virus CNU/LP2.

<6-3> Comparison of viral protein level by Western blot analysis

The present inventors compared viral protein expressed in BHK-21 cells infected with the synthetic JEVs recovered from full-length infectious cDNAs (pBAC^{SP6}/JVFL/*Xho*I, pBAC^{SP6}/JVFLx/*Xho*I, pBAC^{SP6}/JVFLx/*Xba*I, and pBAC^{SP6}/JVFLx/*Xba*I^{MBN}) with that in BHK-21 cells infected with the parental virus CNU/LP2. Particularly, BHK-21 cells ($3x10^5$) were lysed with 200 ㎕ of sample loading buffer [80 mM Tri-HCl (pH 6.8), 2.0% SDS, 10% glycerol, 0.1 M DTT, 0.2% bromophenol blue], and one-tenth of the lysate was boiled for 5 min and fractionated on an SDS-polyacrylamide gel. Proteins were transferred electrophoretically onto a methanol-activated polyvinylidene difluoride membrane with a Trans-Blot SD electrophoretic transfer cell machine (Bio-Rad Laboratories Inc., Hercules, CA), and the membrane was blocked at room temperature for 1 hr with 5% nonfat dried milk in washing solution (0.2% Tween 20 in PBS). After three washes with washing solution, membranes were incubated at room temperature for 2 hr with either a monoclonal anti-actin antibody (A4700,

Sigma, St. Louis, MO) that recognizes the epitope conserved in the C terminus of all actin isoforms or mouse hyperimmune ascites fluid specific for JEV (ATCC VR-1259AF, American Type Culture Collection). The membranes were then washed three times with washing solution and incubated at room temperature for 2 hr with alkaline phosphatase (AP)-conjugated goat anti-mouse immunoglobulin G (Jackson ImmunoResearch Labs Inc., West Grove, PA). The membranes were washed three times with washing solution and once with PBS. Actin and JEV protein bands were visualized by incubation with the substrates 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium. As a result, it was demonstrated that the synthetic JEVs and the parental virus produced similar amounts and identical patterns of virus-specific proteins (FIG. 5C, top panel). Actin protein was measured as an internal sample loading control and revealed equivalent levels of actin protein in mock-infected and infected cells (FIG. 5C, bottom panel).

<6-4> Comparison of viral RNA level by Northern blot analysis

The present inventors compared viral RNA expressed in BHK-21 cells infected with the synthetic

JEVs recovered from full-length infectious cDNAs (pBAC^{SP6}/JVFL/*Xho*I, pBAC^{SP6}/JVFLx/*Xho*I, pBAC^{SP6}/JVFLx/*Xba*I, and pBAC^{SP6}/JVFLx/*Xba*I^{MBN}) with that in BHK-21 cells infected with the parental virus CNU/LP2. Particularly, total RNA was extracted from infected BHK-21 cells ($3x10^5$) with 1 Mℓ of TRIzol reagent (Gibco/BRL). One-third of the RNA was analyzed for JEV-specific RNA by Northern blot analysis (Sambrook et al., *Molecular cloning*, 1989, Cold Spring Harbor Laboratory). The RNA was electrophoresed in denaturing 2.2 M formaldehyde-1% agarose gels and transferred onto nylon membranes (Amersham Biosciences Inc., Piscataway, NJ). The RNA on the membranes was cross-linked by irradiation with a 254-nm light source (Stratalinker UV cross-linker, Stratagene, La Jolla, CA), and the JEV-specific RNAs were detected by hybridization with a [^{32}P]CTP-labeled antisense riboprobe that binds to nt 9,143 to 9,351 of the JEV genome. This probe had been synthesized by *in vitro* transcription from the *Bam*H I-linearized cDNA clone pGEM3Zf(+)/JV9143, which was constructed by ligating the 209-bp *Hind* III-*Sac* I fragment of pBAC^{SP6}/JVFLx/*Xba*I with pGEM3Zf(+) digested with the same enzymes. This clone was transcribed with the T7-MEGAscript kit (Ambion, Austin, TX) as recommended by the manufacturer with a 20-μℓ reaction mixture

containing 3.12 μM [α-^{32}P]CTP(800 Ci/mmol, Amersham). After being treated with DNase I, the reaction mixture was spun in a Quick Spin G-50 Sephadex column (Boehringer Mannheim) to remove unincorporated ribonucleoside triphosphates. The membrane was prehybridized at 55℃ for 6 hr in hybridization solution [5x SSPE(0.9 M NaCl, 50 mM NaH_2PO_4, and 5 mM EDTA pH 7.7), 5x Denhardt's reagent, 0.5% SDS, 100 μg/ml of denatured salmon sperm DNA, 50% formamide] and then incubated at 55℃ overnight in the hybridization solution containing 10^7 cpm of the labeled riboprobe. The membrane was washed three times at 55℃ for 10 min with 1x SSPE-0.5% SDS and once for 10 min with 0.1x SSPE-0.5% SDS. Viral RNA bands were visualized by autoradiography and quantified with a Molecular Imager (Bio-Rad Lab). As a result, viral RNA levels were all similar (FIG. 5D). Quantification of these blots by image analysis revealed that the ratios of viral genomic RNA (FIG. 5D, top panel) to 18S rRNA (FIG. 5D, bottom panel) did not differ significantly, demonstrating that all viral genomic RNAs were produced at similar levels.

Thus, all the synthetic viruses recovered from full-length infectious cDNAs (pBAC^{SP6}/JVFL/*Xho*I, pBAC^{SP6}/JVFLx/*Xho*I, pBAC^{SP6}/JVFLx/*Xba*I, and

pBAC^{SP6}/JVFLx/*Xba*I^{MBN}) were indistinguishable from the parental virus CNU/LP2 in terms of plaque morphology, cytopathogenicity, growth kinetics, protein expression, and RNA production. Furthermore, analyses of the 3′ end sequence did not reveal an extra three (CGA) or four (CTAG) nucleotides of virus-unrelated sequence at the 3′ end of the viral RNA genomes derived from any of the synthetic viruses. These results validate the use of infectious JEV cDNA clones developed in the present invention for future molecular genetics.

Example 7: Check the possibility that the transfected cultures were contaminated with the parental virus

While the above results strongly suggest that the JEV cDNA clones can produce highly infectious RNA transcripts after SP6 or T7 polymerase transcription, the possibility that the transfected cultures were contaminated with the parental virus CNU/LP2 was not formally excluded. To assess this remote possibility, the present inventors used PCR-based site-directed mutagenesis to introduce a genetic marker (gm) into the pBAC^{SP6}/JVFLx/*Xba*I construct. Particularly, the point mutation $A^{8171} \rightarrow C$ (silent) was placed inside the NS5 gene in pBAC^{SP6}/JVFLx/*Xba*I by PCR-based site-directed

mutagenesis to generate pBAC^{SP6}/JVFLx/gm/*Xba*I (FIG. 6A). The point mutation resulted in the acquisition of a unique *Xho* I restriction endonuclease recognition site. A fragment from pBAC^{SP6}/JVFLx/*Xba*I was first generated by PCR with primer J48 represented by SEQ. ID. No 29, in which the *Xho* I was created by the $A^{8171} \rightarrow C$ substitution, and primer J3 represented by SEQ. ID. No 30. The 665-bp *Mlu* I-*Apa* I fragment of the resulting amplicons was then ligated with the 4,802-bp *Apa* I-*Bsr*G I and the 5,874-bp *Bsr*G I-*Mlu* I fragments of pBAC^{SP6}/JVFLx/*Xba*I, resulting in the pBAC^{SP6}/JVFLx/gm/*Xba*I construct. BHK-21 cells transfected with RNA transcripts from *Xba* I-linearized MBN-treated pBAC^{SP6}/JVFLx/gm/*Xba*I^{MBN} produced infectious virus containing the genetic marker (denoted JVFLx/gm/*Xba*I^{MBN}) (FIG. 6A). The phenotypic characteristics of JVFLx/gm/*Xba*I^{MBN} did not differ from those of the original virus JVFLx/*Xba*I^{MBN}, indicating that the $A^{8171} \rightarrow C$ substitution did not affect viral replication.

To verify that the JVFLx/gm/*Xba*I^{MBN} virus had been recovered from the cDNA template of pBAC^{SP6}/JVFLx/gm/*Xba*I^{MBN}, the present inventors serially passaged the recovered virus in BHK-21 cells at an MOI of 0.1. The viruses resulted from each passage were

incubated with RNase A and DNase I to avoid the carryover of the input transcript RNA and template plasmid cDNA (Mendez et al., *J. Virol.*, 1998, 72, 4737-4745). Viral RNAs extracted from the JVFLx/gm/*Xba*I^{MBN} and JVFLx/*Xba*I^{MBN} viruses released at passages 1 and 3 were used in RT-PCR to amplify a 2,580-bp product that encompassed the A^{8171} → C substitution (FIG. 6B, lanes 1, 3, and 5). Digestion of the amplified product from JVFLx/gm/*Xba*I^{MBN} with *Xho* I resulted in two fragments of 1,506 and 1,074 bp (FIG. 6B, lanes 2 and 4). On the other hand, the JVFLx/*Xba*I^{MBN}-derived RT-PCR product did not digest with *Xho* I (FIG. 6B, compared lane 5 with lane 6), demonstrating that the A^{8171} → C substitution was indeed present in the JVFLx/gm/*Xba*I^{MBN} virus. Thus, it was confirmed that the recovered virus JVFLx/gm/*Xba*I^{MBN} originated from the full-length infectious cDNA pBAC^{SP6}/JVFLx/gm/*Xba*I^{MBN}.

Example 8: Genetic stability of full-length infectious JEV cDNA

A previous study has shown that constructs containing full-length JEV cDNA frequently acquired stabilizing nonsense mutations in the regions encoding the structural proteins prM and E (Sumiyoshi et al., *J.*

Virol., 1992, 66, 5425-5431). Since studies into the molecular genetics of JEV will indispensably require a reliable infectious JEV molecular clone for manipulation, the present inventors manipulated pBAC^{SP6}/pJVFLx/*Xba*I in several ways and extensively investigated its genetic structure and functional integrity.

Particularly, the genetic structure and functional integrity of the infectious JEV cDNAs were analyzed as follows. *E.coli* strain DH10B was transformed with pBAC^{SP6}/JVFLx/*Xba*I, and two independently derived clones were grown at 37℃ overnight in 10 ㎖ of 2x YT containing 12.5 ㎍/㎖ of chloramphenicol. Cells from these primary cultures were maintained for 9 days by diluting them 10^6-fold every day (Almazan et al., *Proc. Natl. Acad. Sci. USA*, 2000, 97, 5516-5521). In the experimental conditions of the present invention, each passage represented approximately 20 generations, which was consistent with observations made previously (Alamzan et al., *Proc. Natl. Acad. Sci. USA*, 2000, 97, 5516-5521). After the third, sixth, and ninth passages, large-scale preparation of the infectious cDNA plasmid was made by the SDS-alkaline method and purified further by cesium chloride density gradient centrifugation (Sambrook et

al., *Molecular cloning*, 1989, Cold Spring Harbor Laboratory). The genetic structure of the plasmid DNA was monitored by its restriction endonuclease pattern. The plasmids extracted from the two cultures at passage 0, 3, 6 and 9 were examined by restriction enzyme analysis. The restriction enzyme patterns at passages 3, 6 and 9 did not differ visibly from those at passage 0. Thus, within the resolution of agarose gel electrophoresis analysis, the two infectious cDNA clones appeared to be structurally stable.

The functional integrity of the JEV cDNA plasmid was also investigated by measuring the specific infectivities of the synthetic RNAs transcribed from the cDNA template, which was linearized by *Xba* I digestion and MBN treatment. As a result, the infectivity of the RNA transcripts made from the two cDNA clones did not differ between passage 0 and passage 9 (FIG. 7). From the above result, it was confirmed that the infectious JEV cDNA remained functionally stable during serial growth in *E. coli*.

Example 9: Infectious JEV cDNA as a vector for foreign gene expression

As previously described (Burke and Monath,

Flaviviruses, 2001, 1043-1125, Lippincott Williams & Wilkins Publishers), the present inventors found that JEV was able to replicate in a wide variety of eukaryotic cells originating from a number of species, including humans, mice, monkeys, swine, dogs, cats, and hamsters. This suggests that JEV could be useful as a vector for the expression of heterologous genes in a variety of different cells. To test this, two commonly used reporter genes, the *Aequeorea victoria* GFP and the *Photinus pyralis* LUC, were inserted at the beginning of the viral 3'NTR of pBAC^{SP6}/JVFLx/*Xba*I as expression cassettes driven by the IRES element of EMCV (FIG. 8A).

To create the pBAC^{SP6}/JVFLx/GFP/*Xba*I construct (FIG. 8A), a fragment from pBAC^{SP6}/JVFLx/*Xba*I was amplified by PCR with the primer J72 represented by SEQ. ID. No 31 and the primer J73 represented by SEQ. ID. No 32. A fragment was also amplified from pRSGFP-C1 with the primer J74 represented by SEQ. ID. No 33 and the primer J75 represented by SEQ. ID. No 34. These two fragments were fused by the second round of PCR with the primers J72 and J75. The 913-bp *Kpn* I-*Nsi* I fragment of the resulting amplicons was then ligated with the 8,011-bp *Nsi* I-*Pac* I and 11,021-bp *Pac* I-*Kpn* I fragments of pBAC^{SP6}/JVFLx/*Xba*I, resulting in the pBAC^{SP6}/JVFLx/GFP/*Xba*I construct.

To generate the pBAC^{SP6}/JVFLx/LUC/*Xba*I construct (FIG. 8A), a fragment of pBAC^{SP6}/JVFLx/*Xba*I was amplified with the primer J72 and the primer J76 represented by SEQ. ID. No 35. A fragment was also amplified from pACNR/NADLcIn-/LUC (provided by Dr. Charles M. Rice) with the primer J77 represented by SEQ. ID. No 36 and the primer J78 represented by SEQ. ID. No 37. These two fragments were then fused by the second round of PCR with the primers J72 and J78. The 1,801-bp *Kpn* I-*Nsi* I fragment of the resulting amplicons was then ligated with the 8,011-bp *Nsi* I-*Pac* I and 11,021-bp *Pac* I-*Kpn* I fragments of pBAC^{SP6}/JVFLx/*Xba*I, leading to pBAC^{SP6}/JVFLx/LUC/*Xba*I.

To generate pBAC^{SP6}/JVFLx/LUC^{REP-}/*Xba*I (FIG. 8A), which contains an 83-nucleotide deletion (nt 5,581 to 5,663) in the middle of the NS3 gene that results in premature termination of viral translation at nt 5,596, a fragment of pBAC^{SP6}/JVFLx/LUC/*Xba*I was amplified with the primer J89 represented by SEQ. ID. No 38 and the primer J91 represented by SEQ. ID. No 39. A fragment was also amplified from pBAC^{SP6}/JVFLx/LUC/*Xba*I with the primer J92 represented by SEQ. ID. No 40 and the primer J93 represented by SEQ. ID. No 41. These two fragments were then fused by the second round of PCR with the primers J89 and J93. The 3,960-bp *Sfi* I-*Eag* I fragment

of the resulting amplicons was then ligated with the 6,493-bp *Eag* I-*Sfi* I and 10,297-bp *Sfi* I-*Sfi* I fragments of pBAC^{SP6}/JVFLx/LUC/*Xba*I, leading to pBAC^{SP6}/JVFLx/LUC^{REP-}/*Xba*I.

A deletion of 9 to 25 nucleotides exists at the beginning of the viral 3'NTR in CNP/LP2 and three other fully sequenced JEV strains (Williams et al., *J. Gen. Virol.*, 2000, 81, 2471-2480; Nam et al., *Am. J. Trop. Med. Hyg.*, 2001, 65, 388-392; Jan et al., *Am. J. Troop. Med. Hyg.*, 1996, 55, 603-609), suggesting that this may be a good site to insert the foreign genes. Therefore, when BHK-21 cells were transfected with the synthetic RNAs transcribed from pBAC^{SP6}/JVFLx/GFP/*Xba*I and pBAC^{SP6}/JVFLx/LUC/*Xba*I cDNAs, the insertion did not alter the specific infectivity of the synthetic RNA transcripts.

To examine GFP expression, naive BHK-21 cells were transfected with infectious synthetic RNA transcribed from the pBAC^{SP6}/JVFLx/GFP/*Xba*I^{MBN} template and examined by confocal microscopy. Particularly, BHK-21 cells were mock-transfected or transfected with 2 μg of JVFLx/GFP/*Xba*I^{MBN} RNA. Transfected cells ($1x10^5$) were incubated for 30 hr in a four-well chamber slide. Cells were washed twice with PBS, fixed by incubation for 30 min at 25℃ in PBS containing 0.37% (v/v)

formaldehyde, and mounted with 0.2 Mℓ of 80% glycerol. Cells were viewed by confocal microscopy and analyzed. As a result, BHK-21 cells expressing GFP displayed green fluorescence in both the nucleus and the cytoplasm (FIG. 8B, JVFLx/GFP/*Xba*I^{MBN}) because GFP is small enough to permit diffusion between the nucleus and the cytoplasm. As expected, this fluorescence was not observed in mock-transfected cells (FIG. 8B, mock) or in cells transfected with RNA transcripts from pBAC^{SP6}/JVFLx/*Xba*I^{MBN}.

To monitor the induction of LUC over time in a quantitative manner, the present inventors produced not only replication-competent RNA transcripts from pBAC^{SP6}/JVFLx/LUC/*Xba*I^{MBN} but also replication-incompetent RNA transcripts from pBAC^{SP6}/JVFLx/LUC^{REP-}/*Xba*I^{MBN} (FIG. 8A). The pBAC^{SP6}/JVFLx/LUC^{REP-}/*Xba*I^{MBN} template contains an 83-nucleotide deletion (nt 5,581 to nt 5,663) in the middle of the NS3 gene that prematurely terminates viral translation at nt 5,596 (see * in FIG. 8A, pBAC^{SP6}/JVFLx/LUC^{REP-}/*Xba*I^{MBN}).

For the LUC assay, BHK-21 cells ($8x10^6$) were mock-transfected or transfected with 2 μg of JVFLx/LUC/*Xba*I^{MBN} RNA or JVFLx/LUC^{REP-}/*Xba*I^{MBN} RNA. Cells were seeded at a concentration of $6x10^5$ cells/well in a six-well plate and cultivated. At the given time

points, the cells were washed with Ca^{2+}- and Mg^{2+}-free PBS solution and then lysed by adding 0.2 Mℓ of lysis buffer [25 mM Tris-phosphate (pH 7.8), 2 mM DTT, 2 mM 1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid, 10% glycerol, 1% Triton X-100(v/v)] to each well. Cell lysates were incubated for 10 min at room temperature, and cellular debris was then removed by centrifugation. The supernatants were quickly placed at -80℃ for storage until use. To determine the LUC activity, 20 μℓ of the cell lysates was placed in a luminometer tube containing 100 μℓ of LUC assay reagent [20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2{\cdot}5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 μM coenzyme A, 470 μM luciferin (Promega), 530 μM ATP]. The activity was usually measured for 10 sec. Each data point represents the results of three independent experiments.

As a result, in BHK-21 cells transfected with the replication-competent JVFLx/LUC/*Xba*I^{MBN} RNA (FIG. 8C, •), the initial LUC activity 6 hr posttransfection was $2.4x10^3 \pm 0.2x10^3$ relative light units (RLU). This activity was dramatically increased to $5.3x10^4 \pm 0.1x10^4$ RLU 30 hr posttransfection and reached $7.8x10^5 \pm 0.6x10^5$ RLU 54 hr posttransfection, at which point >50% of the cells were dying. In contrast, in BHK-21 cells transfected with the replication-incompetent

JVFLx/LUC^{REP-}/*Xba*I^{MBN} RNA, the initial LUC activity 6 hr posttransfection was $1.9x10^3 \pm 0.4x10^3$ RLU (FIG. 8C, ○), like the JVFLx/LUC/*Xba*I^{MBN}-transfected cells (FIG. 8C, ●), but this activity gradually decreased over time to 16 ± 1.2 RLU at 54 posttransfection, which is at the level of background luminescence of naïve cells (FIG. 8C, ○). Thus, the level of LUC activity expressed over time varied depending on the presence or absence of viral replication.

The present inventors produced full-length infectious recombinant JEV cDNAs having GFP and LUC genes according to the method explained hereinbefore. BHK-21 cells were transfected with JEV RNA transcripts transcribed from the recombinant JEV cDNAs, and then, recombinant JEV JVFLx/GFP/*Xba*I^{MBN} and JVFLx/LUC/*Xba*I^{MBN} containing GFP and LUC genes were recovered from culture supernatants. The expression of GFP and LUC genes in the recombinant JEV was investigated after infecting a variety of animal cell lines (BHK-21, Vero, NIH/3T3, ST, HeLa, MDCK, CRFK, B103, and SHSY-5Y), which have been generally used in the field of biology and medicine, with the virus. As a result, GFP or LUC gene inserted in virus genome was expressed in all cells tested (Table 4). Thus, it was confirmed that recombinant JEV cDNAs, JEV RNA transcripts, and

recombinant JEV viral particles could be effectively used as a vector for expression of foreign heterologous genes in a variety of cell types.

<Table 4>

Expression of GFP and LUC genes engineered in the infectious JEV cDNAs

Cell line	GFP expression[a]	LUC induction[b]
BHK-21	Expressed	Expressed
Vero	Expressed	Expressed
HeLa	Expressed	Expressed
MDCK	Expressed	Expressed
CRFK	Expressed	Expressed
NIH/3T3	Expressed	Expressed
ST	Expressed	Expressed
B103	Expressed	Expressed
SHSY-5Y	Expressed	Expressed

a : Expression of GFP protein was analyzed after infecting cells with recombinant JEV JVFLx/GFP/*Xba*I^{MBN}.

b : Expression of LUC protein was analyzed after infecting cells with recombinant JEV JVFLx/LUC/*Xba*I^{MBN}.

Example 10: Utility of the infectious JEV cDNA for a novel heterologous gene expression system.

The present inventors further investigated the utility of JEV-based expression system in expressing

foreign genes of interest. First, the present inventors engineered the full-length viral genome to express three commonly used and variously sized heterologous reporter genes, namely, an improved version of the *Aequorea victoria* GFP gene (EGFP, 768 bp), the LUC gene from *Photinus pyralis* (1653 bp), and the LacZ (3012 bp) gene (FIG. 9B). The present inventors also introduced the dominant selective marker PAC (600 bp), which facilitates resistance to the drug puromycin (FIG. 9B).

<10-1> Construction and characterization of heterologous gene-encoding infectious recombinant JEVs that are based on the bicistronic full-length infectious JEV cDNA that serves as a BAC.

<10-1-1> Plasmid construction of infectious recombinant JEV vectors

All plasmids were constructed by standard molecular biology protocols (Sambrook et al., *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989) and all regions amplified by PCR were verified by sequencing. All of the recombinant JEV vectors used in the present invention were constructed based on

pBAC^{SP6}/JVFLx/*Xba*I (Yun et al., *J. Virol.*, 2003, 77, 6450-6465), which is designated as pJEV/FL hereinafter (FIG. 9A).

The present inventors constructed a set of four infectious recombinant JEV vectors expressing the LUC, EGFP, LacZ, and PAC genes. pJEV/FL/LUC is identical to the construction designated as pBAC^{SP6}/JVFLx/LUC/*Xba*I hereinbefore in the Example 9 (FIG. 9B). To construct pJEV/FL/LacZ, the 2,409-bp *Kpn* I-*Avr* II fragment of pJEV/FL/LUC was first subcloned into pGEM3Zf(+) which was digested with *Kpn* I and *Xba* I, resulting in pGEM/LUC. The 3,177-bp *Nco* I-*Stu* I fragment of pSinRep3/LacZ (a generous gift from Dr. Charles Rice) was ligated to the 3,935-bp *Nco* I-*Nsi* I (T4 DNA polymerase-treated) fragment of pGEM/LUC, leading to pGEM/LacZ. The 3,873-bp *Kpn* I-*Not* I fragment of pGEM/LacZ was ligated to the 7,456-bp *Not* I-*Pac* I and 11,021-bp *Pac* I-*Kpn* I fragments of pJEV/FL/LUC, creating pJEV/FL/LacZ (FIG. 9B). To facilitate the construction of pJEV/FL/EGFP, the 5,792-bp *Sac* II-*Not* I fragment of pJEV/FL/LacZ was inserted into pRS2, which was digested with the same enzymes, resulting in pRS/LacZ. A fragment of the sequence coding for EGFP was produced by PCR amplification of pEGFP-C1 with the primers EGFPF (represented by SEQ. ID. No 49) and EGFPR

(represented by SEQ. ID. No 50). The 773-bp *Nco* I-*Stu* I portion of the EGFP fragment amplicons was ligated to the 3,241-bp *Eco*R V-*Sac* II and 2,062-bp *Sac* II-*Nco* I fragments of pRS/LacZ, resulting in pRS/EGFP. The 3,406-bp *Sac* II-*Not* I fragment of pRS/EGFP was ligated to the 7,456-bp *Not* I-*Pac* I and 9,102-bp *Pac* I-*Sac* II fragments of pJEV/FL/LUC, leading to pJEV/FL/EGFP (FIG. 9B). To generate pJEV/FL/PAC, a fragment of pACNR/NADLcIns$^-$/PAC (a generous gift from Dr. Charles Rice) was PCR-amplified with primers PACF (represented by SEQ. ID. No 51) and PACR (represented by SEQ. ID. No 52). The 881-bp *Dra* III-*Nsi* I portion of the resulting amplicons was ligated to the 8,011-bp *Nsi* I-*Pac* I, 10,096-bp *Pac* I-*Nde* I, and 842-bp *Nde* I-*Dra* III fragments of pJEV/FL/LUC, resulting in pJEV/FL/PAC (FIG. 9B). An expression cassette driven by the EMCV IRES was inserted at the beginning of the viral 3′ NTR of pJEV/FL (FIG. 9A and 9B).

<10-1-2> Assay for EGFP expression

Cells were seeded in a four-well chamber slide for 36-48 hr posttransfection. After incubation, cells were fixed by being incubated in PBS containing 0.37% (v/v) formaldehyde and then mounted with 0.2 ml 80% glycerol. Cells were observed under a confocal

microscope outfitted with an appropriate filter. The expression of EGFP was also examined by flow cytometric analysis. Particularly, the cells were trypsinized, washed once with PBS, and resuspended in 0.37% (v/v) formaldehyde in PBS, followed by analysis with a FACScan flow cytometer FACSCalibur (Becton Dickinson). Dead cells were excluded by appropriate forward and side light-scattering gates. Ten thousand viable cells were counted.

<10-1-3> β-galactosidase assay

Cells were washed once with PBS, fixed with 0.05% (v/v) glutaraldehyde in PBS for 15 min at room temperature, and carefully washed three times with PBS. The cells were assessed for β-gal activity by being incubated in staining solution [5mM potassium ferricyanide, 5mM potassium ferrocyanide, 2mM $MgCl_2$ in PBS] with 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (Sigma) at 37°C.

<10-1-4> Luciferase assay

Cells were analyzed for LUC activity by using the substrate luciferin (Promega) as described hereinbefore (Yun et al., *J. Virol.*, 2003, 77, 6450-6465). Each experiment was performed in triplicate and the mean

values are presented.

<10-1-5> Puromycin selection

Cells were seeded in 6-well plates at 37°C for 6 hr. To measure Pur^R foci formation, the cells were overlaid with 0.5% SeaKem LE agarose (FMC BioProducts, Rockland, Maine) in MEM containing 10% heat-inactivated FBS and penicillin/streptomycin and incubated at 37°C for 2 days. Thereafter, the plates were incubated for an additional 3 days in the absence or presence of puromycin (10 μg/ml). After the selection, the Pur^R foci were visualized by crystal violet staining of the formaldehyde-fixed cells (Yun et al., *J. Virol.*, 2003, 77, 6450-6465). For Pur^R cell culture, the cells were left unplugged with the agarose and incubated in complete medium at 37°C for 2 days. Subsequently, the cells were cultivated in complete media containing 10 μg/ml puromycin and 24-48 hr after selection, the surviving cells were visualized by staining with crystal violet.

<10-1-6> Heterologous proteins are expressed in BHK-21 cells transfected/infected with recombinant synthetic JEV RNAs/viruses containing an additional expression unit

To examine whether the insertion of the expression cassette altered its specific infectivity/replication, the present inventors examined the specific *in vitro* infectivity of the synthetic RNAs that had been transcribed from the four SP6-driven foreign gene-bearing infectious JEV cDNA constructs (Table 5). Purified pJEV/FL and its derivative plasmids were linearized by digestion with *Xba* I followed by treatment with MBN. The linearized plasmids were used *in vitro* transcription reactions (25 μℓ) employing SP6 RNA polymerase, as described hereinbefore. After transcription, the reaction mixtures were further incubated with 10 U DNase I for 30 min and extracted with phenol-chloroform-isoamylalcohol. RNA yields were quantified on the basis of [^{3}H]UTP incorporation as measured by RNA absorption to DE-81 filter paper (Whatman, Maidstone, UK). RNA (2 μg) was transfected into cells by electroporation as described hereinbefore (Yun et al., *J. Virol.*, 2003, 77, 6450-6465).

The synthetic RNAs derived from pJEV/FL/PAC, pJEV/FL/EGFP, pJEV/FL/LUC, and pJEV/FL/LacZ introduced into susceptible BHK-21 cells had specific infectivities of $3.5x10^6$, $2.5x10^6$, $3.4x10^6$, and $1.1x10^6$ PFU/μg, respectively, which are similar to the infectivity of the parental pJEV/FL ($3.2x10^6$ PFU/μg).

However, the BHK-21 cells transfected with the recombinant synthetic RNAs did form homogeneous smaller plaques than the pJEV/FL-transfected cells (FIG. 9C). This accords with the delayed production of infectious virus (Table 5, Virus titer) and reduced cytopathogenicity (Table 5, CPE) that was observed in the recombinant RNA-transfected cells. The present inventors also showed that the delayed accumulation of the viral proteins (FIG. 9D) in the recombinant RNA-transfected BHK-21 cells correlated with the length of foreign gene that had been inserted (FIG. 9B).

<Table 5>

Specific infectivity of *in vitro* RNA transcripts generated from full-length JEV cDNA derivatives containing various reporter genes and recombinant virus titer

Template used for transcription[a]	Infectivity[b] (PFU/μg of RNA)	Virus titer[c] (PFU/ml)		CPE[d]
		48 hr	72 hr	
pJEV/FL	3.2×10^6	3.0×10^6	5.1×10^5	++++
pJEV/FL/PAC	3.5×10^6	6.2×10^4	4.0×10^5	++
pJEV/FL/EGFP	2.5×10^6	9.0×10^4	2.1×10^5	++
pJEV/FL/LUC	3.4×10^6	2.0×10^4	3.2×10^5	+
pJEV/FL/LUC^{REP-}	0	0	0	-
pJEV/FL/LacZ	1.1×10^6	1.1×10^4	1.3×10^5	+

a : All JEV cDNA templates used for *in vitro* transcription reaction were prepared by linearization

with *Xba* I digestion, which was followed by treatment with MBN.

b : After *in vitro* transcription with SP6 RNA polymerase, samples were used to electroporate BHK-21 cells, and infectious plaque centers were determined (Yun et al., *J. Virol.*, 2003, 77, 6450-6465).

c : Virus titers at 48 hr and 72 hr postelectroporation.

d : Virus-induced CPE was observed after electroporation with RNA transcripts generated from full-length JEV cDNA derivatives. At 24 hr postelectroporation, strong CPE was observed for the parental pJEV/FL as indicated by ++++. For pJEV/FL/PAC and pJEV/FL/EGFP, CPE was observed at 60 hr postelectroporation as indicated by ++. For pJEV/FL/LacZ, clear CPE began to be displayed at 72 hr postelectroporation as indicated by +. - indicates no CPE.

EGFP, LUC, LacZ and PAC expression using infectious JEV cDNA is shown in FIG. 10. The JEV/FL/EGFP RNA-transfected BHK-21 cells showed bright green fluorescence under a fluorescence microscope (FIG. 10A). The green fluorescent cells (—), as determined by flow cytometry analysis, comprised 99.7% of the cells compared to mock-transfected cells (.....) (FIG.

10B). FIG. 10C demonstrates the X-gal staining pattern of the JEV/FL/LacZ RNA-expressing BHK-21 cells. The present inventors also monitored the LUC activity over time of BHK-21 cells that had been transfected with either the replication-competent JEV/FL/LUC RNA (●) or the replication-incompetent JEV/FL/LUC^{REP-} RNA (○), which lacks a section that prematurely terminates viral translation at nt 5596 (*). This demonstrated that the increased viral replication correlated with increased LUC activity (FIG. 10D), as previously described (Yun et al., *J. Virol.*, 2003, 77, 6450-6465). Furthermore, selection of the JEV/FL/PAC RNA-transfected BHK-21 cells with puromycin (FIG. 10E) revealed the JEV/FL/PAC RNA-transfected cells survived and become confluent in the puromycin-containing media (dish 7) or formed Pur^{R} foci under semisolid agar overlaid with puromycin-containing media (dish 8), whereas the mock-transfected cells died within 24 hr of selection (dishes 5-6). As expected, both cell types became confluent in the absence of puromycin (dishes 1-4).

<10-2> Construction and vector characteristics of JEV viral replicons

<10-2-1> Plasmid construction of JEV viral replicon vectors

Plasmids for all JEV viral replicons were constructed based on pJEV/FL/LUC by engineering in-frame deletions in the coding sequences of the structural proteins. All deletions were distinguished by a novel *Xho* I site that resulted in the insertion of two residues, namely, Leu and Glu. First, the present inventors generated a set of four JEV viral replicon vectors containing a single in-frame deletion in each structural protein. To construct pJEV/Rep/ΔCC/LUC, which contains a 273-nucleotide deletion (nt 132-404) in the C gene, two fragments were synthesized by PCR amplification of pJEV/FL, namely, fragment C1 with primers DelF (represented by SEQ. ID. No 53) and C1R (represented by SEQ. ID. No 54), and fragment C2 with primers C2F (represented by SEQ. ID. No 55) and DelR (represented by SEQ. ID. No 56). Two fragments (the 267-bp *Pac* I-*Xho* I portion of the C1 fragment amplicons and the 226-bp *Xho* I-*BsiW* I portion of the C2 fragment amplicons) were ligated to the 20,073-bp *BsiW* I-*Pac* I fragment of pJEV/FL/LUC, resulting in the pJEV/Rep/ΔCC/LUC construct. To generate pJEV/Rep/ΔC/LUC, which contains a 204-nucleotide deletion (nt 201-404) in the C gene, fragment C3 from pJEV/FL was amplified by PCR with the primers DelF and C3R (represented by SEQ. ID. No 57). The 336-bp *Pac* I-

Xho I fragment of the resulting amplicons was ligated to the 12,850-bp *Xho* I-*Rsr* II and 7,449-bp *Rsr* II-*Pac* I fragments of pJEV/Rep/ΔCC/LUC, resulting in the pJEV/Rep/ΔC/LUC construct. To create pJEV/Rep/ΔprM/LUC, which contains a 282-nucleotide deletion (nt 531-812) in the prM gene, two fragments were obtained by the PCR amplification of pJEV/FL, namely, fragment prM1 with the primers DelF and prM1R (represented by SEQ. ID. No 58), and fragment prM2 with primers prM2F (represented by SEQ. ID. No 59) and DelR. Two fragments (the 666-bp *Pac* I-*Xho* I portion of the prM1 fragment amplicons and the 1,616-bp *Xho* I-*Sfi* I portion of the prM2 fragment amplicons) were ligated to the 10,264-bp *Sfi* I-*Nsi* I and 8,011-bp *Nsi* I-*Pac* I fragments of pJEV/FL/LUC, resulting in the pJEV/Rep/ΔprM/LUC construct. To engineer pJEV/Rep/ΔE/LUC, which contains a 1,170-nucleotide deletion (nt 1,032-2,201) in the E gene, two fragments were produced by PCR amplification of pJEV/FL, namely, fragment E1 with primers DelF and E1R (represented by SEQ. ID. No 60), and fragment E2 with primers E2F (represented by SEQ. ID. No 61) and DelR. Two fragments (the 1,167-bp *Pac* I-*Xho* I portion of the prM1 fragment amplicons and the 227-bp *Xho* I-*Sfi* I portion of the prM2 fragment amplicons) were ligated to the 10,264-bp *Sfi* I-*Nsi* I and 8,011-bp *Nsi* I-*Pac* I

fragments of pJEV/FL/LUC, resulting in the pJEV/Rep/ΔE/LUC construct (FIG. 11A).

Second, the present inventors constructed a panel of three JEV viral replicon vectors that contain a double in-frame deletion in the JEV structural genes. Two fragments of pJEV/FL/LUC (the 10,264-bp *Sfi* I-*Nsi* I and 8,011-bp *Nsi* I-*Pac* I fragments) were ligated to either (*i*) the 438-bp *Pac* I-*Hin*d III fragment of pJEV/Rep/ΔC/LUC and the 1,646-bp *Hin*d III-*Sfi* I fragment of pJEV/Rep/ΔprM/LUC to generate pJEV/Rep/ΔC+ΔprM/LUC, (*ii*) the 866-bp *Pac* I-*Mlu* I fragment of pJEV/Rep/ΔC/LUC and the 330-bp *Mlu* I-*Sfi* I fragment of pJEV/Rep/ΔE/LUC to generate pJEV/Rep/ΔC+ΔE/LUC, or (*iii*) the 788-bp *Pac* I-*Mlu* I fragment of pJEV/Rep/ΔprM/LUC and the 330-bp *Mlu* I-*Sfi* I fragment of pJEV/Rep/ΔE/LUC to generate pJEV/Rep/ΔprM+ΔE/LUC (FIG. 11A).

Third, the present inventors created a set of two JEV viral replicon vectors in which all JEV structural proteins were lacking. To generate pJEV/Rep/ΔC+ΔprM+ΔE/LUC, two fragments of pJEV/FL/LUC (the 10,264-bp *Sfi* I-*Nsi* I and 8,011-bp *Nsi* I-*Pac* I fragments) were ligated to the 590-bp *Pac* I-*Mlu* I

fragment of pJEV/Rep/ΔC+ΔprM/LUC and the 330-bp *Mlu* I-*Sfi* I fragment of pJEV/Rep/ΔE/LUC. The present inventors also constructed pJEV/Rep/NS1/LUC, which contains the 35 N-terminal and 24 C-terminal amino acids of the C protein followed immediately by the N-terminus of the NS1 protein and the rest of the viral genome. A fragment from pJEV/Rep/ΔC/LUC was first synthesized by PCR with the primers DelF and NS1R (represented by SEQ. ID. No 62). A fragment from pJEV/FL was then synthesized with the primers NS1F (represented by SEQ. ID. No 63) and RR (represented by SEQ. ID. No 64). These two fragments were fused by a second round of PCR with the primers DelF and RR. The 474-bp *Pac* I-*Apa*L I fragment of the resulting amplicons was ligated to the 3,038-bp *Apa*L I-*Bam*H I and 15,122-bp *Bam*H I-*Pac* I fragments of pJEV/FL/LUC, leading to pJEV/Rep/NS1/LUC (FIG. 11A).

In addition to pJEV/Rep/ΔC+ΔprM+ΔE/LUC and pJEV/Rep/NS1/LUC, the present inventors also constructed eight other JEV viral replicon vectors. The 6,797-bp *Bam*H I-*Not* I fragment of pJEV/FL/EGFP was ligated to either (*i*) the 11,529-bp *Bam*H I-*Not* I fragment of pJEV/Rep/ΔC+ΔprM+ΔE/LUC to create pJEV/Rep/ΔC+ΔprM+ΔE/EGFP, or (*ii*) the 10,968-bp *Bam*H I-

Not I fragment of pJEV/Rep/NS1/LUC to create pJEV/Rep/NS1/EGFP. The 5,792-bp *Sac* II-*Not* I fragment of pJEV/FL/LacZ was ligated to either (*i*) the 7,456-bp *Not* I-*Pac* I and the 7,464-bp *Pac* I-*Sac* II fragments of pJEV/Rep/ΔC+ΔprM+ΔE/LUC to create pJEV/Rep/ΔC+ΔprM+ΔE/LacZ, or (*ii*) the 7,456-bp *Not* I-*Pac* I and the 6,903-bp *Pac* I-*Sac* II fragments of pJEV/Rep/NS1/LUC to create pJEV/Rep/NS1/LacZ. The 6,663-bp *Bam*H I-*Not* I fragment of pJEV/FL/PAC was ligated to either (*i*) the 11,529-bp *Bam*H I-*Not* I fragment of pJEV/Rep/ΔC+ΔprM+ΔE/LUC to create pJEV/Rep/ΔC+ΔprM+ΔE/PAC, or (*ii*) the 10,968-bp *Bam*H I-*Not* I fragment of pJEV/Rep/NS1/LUC to create pJEV/Rep/NS1/PAC.

<10-2-2> Heterologous proteins are expressed from a variety of self-replicating self-limiting JEV viral replicons

To independently express foreign genes using the JEV RNA replication machinery, the present inventors generated a panel of self-replicating self-limiting viral replicons that meet stringent safety concerns (FIG. 11A). Initially, the present inventors used the LUC reporter as the heterologous gene as it facilitates the monitoring of viral replication in a sensitive and

quantitative manner. Thus, a variety of replicon vectors were carefully engineered in the context of pJEV/FL/LUC by the in-frame deletion of one, two, or all of the viral structural genes (C, prM, and E), in consideration with the membrane orientation of each protein (FIG. 11A).

The LUC activities of the BHK-21 cells that had been transfected with the various viral replicons were plotted over time (FIG. 11B). In BHK-21 cells transfected with the replication-competent JEV/FL/LUC RNA (● , black) as a positive control, the initial LUC activity at 6 hr posttransfection was 5.5±0.3 X 10^3 RLU. This activity dramatically increased to 2.7±0.5 X 10^6 RLU at 48 hr posttransfection and was maintained through to 96 hr posttransfection. In BHK-21 cells transfected with the replication-incompetent JEV/FL/LUC^{REP-} RNA (◆ , black), the initial LUC activity expressed from the input viral RNA at 6 hr posttransfection was similar, namely, 5.2±0.6 X 10^3 RLU. However, this activity gradually decreased over time to 8.8±1.0 RLU at 96 hr posttransfection, which is equivalent to the background luminescence of naïve cells. Apart from pJEV/Rep/ΔCC/LUC (◆ , blue), which lacks a sequence that is complementary to a proposed cyclization sequence in the 3'NTR that is conserved in

all flaviviruses (Bredenbeek et al., *J. Gen. Virol.*, 2003, 84, 1261-1268; Lo et al., *J. Virol.*, 2003, 77, 10004-10014; Khromykh et al., *J. Virol.*, 2001, 75, 6719-6728), the LUC activities of the BHK-21 cells transfected with the viral replicons lacking part of one or more structural protein genes were almost identical in the 6-48 hrs posttransfection to those of the replication-competent JEV/FL/LUC RNA-transfected BHK-21 cells (● , black). Thereafter, however, these activities decreased dramatically over time due to a lack of viral spread, similar to JEV/FL/LUC^{REP-}. Interestingly, the LUC activities due to JEV/Rep/NS1/LUC RNA (● , green), but not to JEV/Rep/ΔC+ΔprM+ΔE/LUC RNA (■ , green), were approximately 5-fold higher at all time points compared to the activities of the other replication-competent viral replicons.

The LUC expression profiles agreed with the viral protein accumulation (FIG. 11C), as quantified by immunoblotting with JEV-specific hyperimmune sera. The present inventors also confirmed that other reporter genes could be efficiently expressed in various commonly used animal cells by using JEV-based replicon vectors such as pJEV/Rep/NS1 and pJEV/Rep/ΔC+ΔprM+ΔE.

<10-3> Construction of the packaging system for JEV viral replicons

<10-3-1> Plasmid construction of JEV structural protein expression vectors based on the pSinRep19 vector

The present inventors constructed three JEV structural protein expression vectors based on pSinRep19 (Agapov et al., *Proc. Natl. Acad. Sci. USA*, 1998, 95, 12989-12994). For pSinRep19/JEV C-E, a fragment of pJEV/FL was amplified with primer JEVCF (5'-GATTCTAGAATGACTAAAAAACCA, represented by SEQ. ID. No 65), which incorporates an *Xba* I site (underlined) and primer JEVER (5'-GATGTTTAAACTATTAAGCATGCACATTGGT, represented by SEQ. ID. No 66), which incorporates a *Pme* I site (underlined). The 2,393-bp *Xba* I-*Pme* I fragment of the resulting amplicons was ligated to the 10,864-bp *Xba* I-*Mlu* I (T4 DNA polymerase-treated) fragment of pSinRep19 to construct pSinRep19/JEV C-E (FIG. 12A). For pSinRep19/JEV C-NS1, a fragment was obtained by PCR amplification of pJEV/FL with primer JEVCF and primer JEVNS1R (5'-GATGTTTAAACTATTAAGCATCAACCTGTGA, represented by SEQ. ID. No 67), which incorporates a *Pme* I site (underlined). The 3,449-bp *Xba* I-*Pme* I fragment of the resulting amplicons was then ligated to the 10,864-bp *Xba* I-*Mlu* I

(T4 DNA polymerase-treated) fragment of pSinRep19 to construct pSinRep19/JEV C-NS1 (FIG. 12A). For pSinRep19/JEV C-E-*Bgl*II, the 2,559-bp *Xba* I-*Bgl* II (T4 DNA polymerase-treated) fragment of pSinRep19/JEV C-NS1 was ligated to the 10,864-bp *Xba* I-*Mlu* I (T4 DNA polymerase-treated) fragment of pSinRep19 (FIG. 12A).

<10-3-2> Generation of packaging cell lines for JEV-derived replicon vector RNAs.

The utility of the JEV replicon-based expression vectors was elaborated by developing packaging cell lines (PCLs) that constitutively express all the structural proteins of JEV (C, prM, and E) and allow the *trans*-complementation of the efficient packaging of JEV viral replicons. Based on the pSinRep19 expression vector that contains the PAC gene driven by the subgenomic promoter, which facilitates selection (FIG. 12A), the present inventors constructed three different JEV structural protein expression cassette constructs that encode the sequences for C-E (pSinRep19/JEV C-E), C-E plus the 58 N-terminal residues of NS1 (pSinRep19/JEV C-E-*Bgl*II), and C-NS1 (pSinRep19/JEV C-NS1).

The protein expression yielded by these vectors

was evaluated in BHK-21 cells transfected with the synthetic RNAs that had been transcribed *in vitro* from the corresponding vector. pSinRep19 and its derivatives were linearized by digestion with *Xho* I. The linearized plasmids were used *in vitro* transcription reactions (25 μℓ) employing SP6 RNA polymerase, as described hereinbefore. After transcription, the reaction mixtures were further incubated with 10 U DNase I for 30 min and extracted with phenol-chloroform-isoamylalcohol. RNA yields were quantified on the basis of [^{3}H]UTP incorporation as measured by RNA absorption to DE-81 filter paper (Whatman, Maidstone, UK). RNA (2 μg) was transfected into cells by electroporation as described hereinbefore (Yun et al., *J. Virol.*, 2003, 77, 6450-6465). When the cell lysates from the transfected cells were analyzed by immunoblotting with JEV-specific hyperimmune sera, equal amounts of viral glycoprotein E were detected in the BHK-21 cells transfected with each of the three vectors (FIG. 12B). As designed, the NS1 protein was detected only in the SinRep19/JEV C-NS1 RNA-transfected cells (FIG. 12B).

Two approaches to produce JEV viral replicon particles (VRPs) are illustrated in FIG. 12C. One

involves the transient cotransfection of *in vitro*-transcribed JEV replicon vector RNA with the SinRep19 vector RNA that expresses the JEV structural proteins. Titering and monitoring of the packaged VRPs was made possible by infecting naïve BHK-21 cells with the VRPs and then assaying for reporter gene expression. Cotransfection of SinRep19/JEV C-NS1 RNA with EGFP-expressing JEV viral replicon RNAs [either JEV/Rep/ΔC+ΔprM+ΔE/EGFP (□, green) or JEV/Rep/NS1/EGFP (■, green)] in several experiments produced 1.1-4.3X10^4 infectious units/ml (IU/ml) of VRPs (FIG. 12D). Similar results were obtained using LacZ-expressing JEV viral replicons, namely, either JEV/Rep/ΔC+ΔprM+ΔE/LacZ (□, blue) or JEV/Rep/NS1/LacZ (■, blue). No difference was observed when the SinRep19/JEV C-NS1 or the SinRep19/JEV C-E-*Bgl*II JEV structural protein expression cassettes were used. However, cotransfection of the SinRep19/JEV C-E vector RNA with the viral replicons expressing either EGFP or LacZ produced ≈ 100-fold fewer VRPs (FIG. 12D). These observations were confirmed by cotransfecting all JEV structural protein expression vector RNAs with the LUC-expressing JEV replicon RNAs, namely, JEV/Rep/ΔC+ΔprM+ΔE/LUC (□, black) or JEV/Rep/NS1/LUC (■, black) (FIG. 12D).

The other approach to producing JEV VRPs is based on using a continuous PCL, which is established by transfecting cells with the JEV structural protein expression vector RNA and selecting with puromycin. The BHK-21 cells were transfected with JEV structural protein expression vector RNAs as mentioned hereinbefore. After transfection, the cells were seeded for ≈24 hr and the media were replaced with fresh complete media containing 10 μg/ml puromycin (Sigma). Thereafter, the cells were maintained in the presence of puromycin and passaged or frozen as the parental BHK-21 cells.

The selected cells were shown to stably express the JEV structural proteins without any deleterious effects to the host cell and were slightly more efficient in producing JEV-based VRPs than the parental BHK-21 cells. In all cases, higher VRP titers ($1.0X10^3$-$1.2X10^5$ IU/ml) were obtained upon transfection of these PCLs with the JEV viral replicon vector RNAs, as compared to the protocol involving the cotransfection of the parental BHK-21 cells with two vector RNAs (FIG. 12E).

To test for the presence of replication-competent viral particles in the packaging system developed in

the present invention, naïve BHK-21 cells were infected with $3X10^5$ IU of the VRPs at an MOI of 1 for 72 hr. The undiluted supernatant obtained from the infected cells was further passaged three times to amplify the possible existence of very low amounts of replication-competent viral particles. At the end of these passages, the infected cells were tested for the expression of the reporter gene or viral protein by IFA using JEV-specific hyperimmune sera. No replication-competent viral particles were ever detected. Furthermore, Sindbis replicon RNAs that express JEV structural proteins were not encapsidated in the released VRPs.

INDUSTRIAL APPLICABILITY

As explained hereinbefore, the authentic nucleotide sequence of JEV genomic RNA and the full-length infectious JEV cDNA of the present invention synthesized therefrom can be used not only for the identification of the JEV genes, but also for the molecular biological studies including JEV replication, transcription, and translation. Moreover, they can also be applied to the development of the therapeutic

agents, vaccines, diagnostic reagents, and diagnostic devices for Japanese encephalitis, and can be used as an expression vector for the various foreign genes.

Those skilled in the art will appreciate that the conceptions and specific embodiments disclosed in the foregoing description may be readily utilized as a basis for modifying or designing other embodiments for carrying out the same purposes of the present invention. Those skilled in the art will also appreciate that such equivalent embodiments do not depart from the spirit and scope of the invention as set forth in the appended claims.

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO : LEE, Young-Min
College of Medicine, Chungbuk National University,
#48, Gaeshin-dong, Heungduk-ku, Cheongju-si, Chungbuk 361 763,
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: **pBAC^{T7}/JVFLx/XbaI (plasmid)**	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: **KCTC 10346BP**

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION
The microorganism identified under I above was accompanied by: [x] a scientific description [ ] a proposed taxonomic designation (Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on **October 02 2002.**

IV. RECEIPT OF REQUEST FOR CONVERSION
The microorganism identified under I above was received by this International Depositary Authority on and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on

V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: **Korean Collection for Type Cultures** Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority of authorized official(s): PARK Yong-Ha, Director Date: **October 04 2002**

Form BP/4 (KCTC Form 17)

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

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RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

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TO : LEE, Young-Min
College of Medicine, Chungbuk National University,
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Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: **pBAC^{SP6}/JVFLx/XbaI (plasmid)**	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: **KCTC 10347BP**

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:
[x] a scientific description
[ ] a proposed taxonomic designation
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Form BP/4 (KCTC Form 17)